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<p>(21) International Application Number: <b>PCT/US97/02219</b></p> <p>(22) International Filing Date: <b>10 February 1997 (10.02.97)</b></p> <p>(30) Priority Data:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">08/599,226</td> <td style="width: 40%;">9 February 1996 (09.02.96)</td> <td style="width: 20%; text-align: right;">US</td> </tr> <tr> <td>60/031,476</td> <td>25 November 1996 (25.11.96)</td> <td style="text-align: right;">US</td> </tr> </table> <p>(60) Parent Applications or Grants</p> <p>(63) Related by Continuation</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">US</td> <td style="width: 40%;">08/599,226 (CIP)</td> <td style="width: 20%;"></td> </tr> <tr> <td>Filed on</td> <td>9 February 1996 (09.02.96)</td> <td></td> </tr> <tr> <td>US</td> <td>60/031,476 (CIP)</td> <td></td> </tr> <tr> <td>Filed on</td> <td>25 November 1996 (25.11.96)</td> <td></td> </tr> </table> <p>(71) Applicant (for all designated States except US): <b>BASF AKTIENGESELLSCHAFT [DE/DE]; Carl-Bosch-Strasse 38, D-67056 Ludwigshafen (DE).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): <b>SALFELD, Jochen, G. [DE/US]; 177 Old Westboro Road, North Grafton, MA 01536 (US). ALLEN, Deborah, J. [GB/GB]; 143a Shelbourne Road, London N17 9YD (GB). KAYMAKALAN, Zehra [TR/US]; 4 Piccadilly Way, Westboro, MA 01581</b></p>			08/599,226	9 February 1996 (09.02.96)	US	60/031,476	25 November 1996 (25.11.96)	US	US	08/599,226 (CIP)		Filed on	9 February 1996 (09.02.96)		US	60/031,476 (CIP)		Filed on	25 November 1996 (25.11.96)	
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<p>(54) Title: <b>HUMAN ANTIBODIES THAT BIND HUMAN TNF<math>\alpha</math></b></p> <p>(57) Abstract</p> <p>Human antibodies, preferably recombinant human antibodies, that specifically bind to human tumor necrosis factor <math>\alpha</math> (hTNF<math>\alpha</math>) are disclosed. These antibodies have high affinity for hTNF<math>\alpha</math> (e.g., <math>K_d = 10^{-8}</math> M or less), a slow off rate for hTNF<math>\alpha</math> dissociation (e.g., <math>K_{off} = 10^{-3}</math> sec<sup>-1</sup> or less) and neutralize hTNF<math>\alpha</math> activity <i>in vitro</i> and <i>in vivo</i>. An antibody of the invention can be a full-length antibody or an antigen-binding portion thereof. The antibodies, or antibody portions, of the invention are useful for detecting hTNF<math>\alpha</math> and for inhibiting hTNF<math>\alpha</math> activity, e.g., in a human subject suffering from a disorder in which hTNF<math>\alpha</math> activity is detrimental. Nucleic acids, vectors and host cells for expressing the recombinant human antibodies of the invention, and methods of synthesizing the recombinant human antibodies, are also encompassed by the invention.</p>																				

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HUMAN ANTIBODIES THAT BIND HUMAN TNF $\alpha$ **Background of the Invention**

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is a cytokine produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see *e.g.*, Old, L. (1985) *Science* 230:630-632). Subsequently, a factor termed cachectin, associated with cachexia, was shown to be the same molecule as TNF $\alpha$ . TNF $\alpha$  has been implicated in mediating shock (see *e.g.*, Beutler, B. and Cerami, A. (1988) *Annu. Rev. Biochem.* 57:505-518; Beutler, B. and Cerami, A. (1989) *Annu. Rev. Immunol.* 7:625-655). Furthermore, TNF $\alpha$  has been implicated in the pathophysiology of a variety of other human diseases and disorders, including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease (see *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A., *et al.* Vasilli, P. (1992) *Annu. Rev. Immunol.* 10:411-452; Tracey, K.J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503).

Because of the harmful role of human TNF $\alpha$  (hTNF $\alpha$ ) in a variety of human disorders, therapeutic strategies have been designed to inhibit or counteract hTNF $\alpha$  activity. In particular, antibodies that bind to, and neutralize, hTNF $\alpha$  have been sought as a means to inhibit hTNF $\alpha$  activity. Some of the earliest of such antibodies were mouse monoclonal antibodies (mAbs), secreted by hybridomas prepared from lymphocytes of mice immunized with hTNF $\alpha$  (see *e.g.*, Hahn T; *et al.*, (1985) *Proc Natl Acad Sci USA* 82: 3814-3818; Liang, C-M., *et al.* (1986) *Biochem. Biophys. Res. Commun.* 137:847-854; Hirai, M., *et al.* (1987) *J. Immunol. Methods* 96:57-62; Fendly, B.M., *et al.* (1987) *Hybridoma* 6:359-370; Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 186 833 B1 by Wallach, D.; European Patent Application Publication No. 218 868 A1 by Old *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A., *et al.*). While these mouse anti-hTNF $\alpha$  antibodies often displayed high affinity for hTNF $\alpha$  (*e.g.*, K<sub>d</sub>  $\leq$  10<sup>-9</sup>M) and were able to neutralize hTNF $\alpha$  activity, their use *in vivo* may be limited by problems associated with administration of mouse antibodies to humans, such as short serum half life, an inability to trigger certain human effector functions and elicitation of an unwanted immune response against the mouse antibody in a human (the "human anti-mouse antibody" (HAMA) reaction).

In an attempt to overcome the problems associated with use of fully-murine antibodies in humans, murine anti-hTNF $\alpha$  antibodies have been genetically engineered

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to be more "human-like." For example, chimeric antibodies, in which the variable regions of the antibody chains are murine-derived and the constant regions of the antibody chains are human-derived, have been prepared (Knight, D.M, *et al.* (1993) *Mol. Immunol.* 30:1443-1453; PCT Publication No. WO 92/16553 by Daddona, P.E., *et al.*).

5 Additionally, humanized antibodies, in which the hypervariable domains of the antibody variable regions are murine-derived but the remainder of the variable regions and the antibody constant regions are human-derived, have also been prepared (PCT Publication No. WO 92/11383 by Adair, J.R., *et al.*). However, because these chimeric and humanized antibodies still retain some murine sequences, they still may elicit an

10 unwanted immune reaction, the human anti-chimeric antibody (HACA) reaction, especially when administered for prolonged periods, *e.g.*, for chronic indications, such as rheumatoid arthritis (see *e.g.*, Elliott, M.J., *et al.* (1994) *Lancet* 344:1125-1127; Elliot, M.J., *et al.* (1994) *Lancet* 344:1105-1110).

A preferred hTNF $\alpha$  inhibitory agent to murine mAbs or derivatives thereof (*e.g.*, chimeric or humanized antibodies) would be an entirely human anti-hTNF $\alpha$  antibody, since such an agent should not elicit the HAMA reaction, even if used for prolonged periods. Human monoclonal autoantibodies against hTNF $\alpha$  have been prepared using human hybridoma techniques (Boyle, P., *et al.* (1993) *Cell. Immunol.* 152:556-568; Boyle, P., *et al.* (1993) *Cell. Immunol.* 152:569-581; European Patent Application

20 Publication No. 614 984 A2 by Boyle, *et al.*). However, these hybridoma-derived monoclonal autoantibodies were reported to have an affinity for hTNF $\alpha$  that was too low to calculate by conventional methods, were unable to bind soluble hTNF $\alpha$  and were unable to neutralize hTNF $\alpha$ -induced cytotoxicity (see Boyle, *et al.*; *supra*). Moreover, the success of the human hybridoma technique depends upon the natural presence in

25 human peripheral blood of lymphocytes producing autoantibodies specific for hTNF $\alpha$ . Certain studies have detected serum autoantibodies against hTNF $\alpha$  in human subjects (Fomsgaard, A., *et al.* (1989) *Scand. J. Immunol.* 30:219-223; Bendtzen, K., *et al.* (1990) *Prog. Leukocyte Biol.* 10B:447-452), whereas others have not (Leusch, H-G., *et al.* (1991) *J. Immunol. Methods* 139:145-147).

30 Alternative to naturally-occurring human anti-hTNF $\alpha$  antibodies would be a recombinant hTNF $\alpha$  antibody. Recombinant human antibodies that bind hTNF $\alpha$  with relatively low affinity (*i.e.*,  $K_d \sim 10^{-7}M$ ) and a fast off rate (*i.e.*,  $K_{off} \sim 10^{-2} \text{ sec}^{-1}$ ) have been described (Griffiths, A.D., *et al.* (1993) *EMBO J.* 12:725-734). However, because of their relatively fast dissociation kinetics, these antibodies may not be suitable for

35 therapeutic use. Additionally, a recombinant human anti-hTNF $\alpha$  has been described that does not neutralize hTNF $\alpha$  activity, but rather enhances binding of hTNF $\alpha$  to the

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surface of cells and enhances internalization of hTNF $\alpha$  (Lidbury, A., *et al.* (1994) *Biotechnol. Ther.* 5:27-45; PCT Publication No. WO 92/03145 by Aston, R. *et al.*)

Accordingly, human antibodies, such as recombinant human antibodies, that bind soluble hTNF $\alpha$  with high affinity and slow dissociation kinetics and that have the capacity to neutralize hTNF $\alpha$  activity, including hTNF $\alpha$ -induced cytotoxicity (*in vitro* and *in vivo*) and hTNF $\alpha$ -induced cell activation, are still needed.

### Summary of the Invention

This invention provides human antibodies, preferably recombinant human antibodies, that specifically bind to human TNF $\alpha$ . The antibodies of the invention are characterized by binding to hTNF $\alpha$  with high affinity and slow dissociation kinetics and by neutralizing hTNF $\alpha$  activity, including hTNF $\alpha$ -induced cytotoxicity (*in vitro* and *in vivo*) and hTNF $\alpha$ -induced cellular activation. Antibodies of the invention are further characterized by binding to hTNF $\alpha$  but not hTNF $\beta$  (lymphotoxin) and by having the ability to bind to other primate TNF $\alpha$ s and non-primate TNF $\alpha$ s in addition to human TNF $\alpha$ .

The antibodies of the invention can be full-length (*e.g.*, an IgG1 or IgG4 antibody) or can comprise only an antigen-binding portion (*e.g.*, a Fab, F(ab')<sub>2</sub> or scFv fragment). The most preferred recombinant antibody of the invention, termed D2E7, has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 and a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4. Preferably, the D2E7 antibody has a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2.

In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNF $\alpha$  with a  $K_d$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant of  $1 \times 10^{-3}$  s<sup>-1</sup> or less, both determined by surface plasmon resonance, and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an IC<sub>50</sub> of  $1 \times 10^{-7}$  M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $5 \times 10^{-4}$  s<sup>-1</sup> or less, or even more preferably, with a  $K_{off}$  of  $1 \times 10^{-4}$  s<sup>-1</sup> or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an IC<sub>50</sub> of  $1 \times 10^{-8}$  M or less, even more preferably with an IC<sub>50</sub> of  $1 \times 10^{-9}$  M or less and still more preferably with an IC<sub>50</sub> of  $5 \times 10^{-10}$  M or less.

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In another embodiment, the invention provides a human antibody, or antigen-binding portion thereof, with the following characteristics:

- a) dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance;
- 5        b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;
- 10        c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $5 \times 10^{-4} \text{ s}^{-1}$  or less. Still more preferably, the  
15    antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $1 \times 10^{-4} \text{ s}^{-1}$  or less.

In yet another embodiment, the invention provides a human antibody, or an antigen-binding portion thereof, with an LCVR having CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single  
20    alanine substitution at position 1, 4, 5, 7 or 8, and with an HCVR having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. More preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 and the HCVR further has a CDR2 domain comprising the amino acid  
25    sequence of SEQ ID NO: 6. Still more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with an LCVR comprising the amino acid  
30    sequence of SEQ ID NO: 1 and an HCVR comprising the amino acid sequence of SEQ ID NO: 2. In certain embodiments, the antibody has an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. In yet other embodiments, the antibody is a Fab fragment, an F(ab')<sub>2</sub> fragment or a single chain Fv fragment.

In still other embodiments, the invention provides antibodies, or antigen-binding  
35    portions thereof, with an LCVR having CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID

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NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or with an HCVR having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, that neutralizes the activity of human TNF $\alpha$  but not human TNF $\beta$  (lymphotoxin). In a preferred embodiment, the human antibody, or antigen-binding portion thereof, neutralizes the activity of human TNF $\alpha$ , chimpanzee TNF $\alpha$  and at least one additional primate TNF $\alpha$  selected from the group consisting of baboon TNF $\alpha$ , marmoset TNF $\alpha$ , cynomolgus TNF $\alpha$  and rhesus TNF $\alpha$ . Preferably, the antibody also neutralizes the activity of at least one non-primate TNF $\alpha$ . For example, in one subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of canine TNF $\alpha$ . In another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of pig TNF $\alpha$ . In yet another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of mouse TNF $\alpha$ .

Another aspect of the invention pertains to nucleic acid molecules encoding the antibodies, or antigen-binding portions, of the invention. A preferred nucleic acid of the invention, encoding a D2E7 LCVR, has the nucleotide sequence shown in Figure 7 and SEQ ID NO 36. Another preferred nucleic acid of the invention, encoding a D2E7 HCVR, has the nucleotide sequence shown in Figure 8 and SEQ ID NO 37.

Recombinant expression vectors carrying the antibody-encoding nucleic acids of the invention, and host cells into which such vectors have been introduced, are also encompassed by the invention, as are methods of making the antibodies of the invention by culturing the host cells of the invention.

Yet another aspect of the invention pertains to methods for inhibiting human TNF $\alpha$  activity using an antibody, or antigen-binding portion thereof, of the invention. In one embodiment, the method comprises contacting human TNF $\alpha$  with the antibody of the invention, or antigen-binding portion thereof, such that human TNF $\alpha$  activity is inhibited. In another embodiment, the method comprises administering an antibody of the invention, or antigen-binding portion thereof, to a human subject suffering from a disorder in which TNF $\alpha$  activity is detrimental such that human TNF $\alpha$  activity in the human subject is inhibited. The disorder can be, for example, sepsis, an autoimmune

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disease (e.g., rheumatoid arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome), an infectious disease, a malignancy, transplant rejection or graft-versus-host disease, a pulmonary disorder, a bone disorder, an intestinal disorder or a cardiac disorder.

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### **Brief Description of the Drawings**

Figures 1A and 1B show the amino acid sequences of the light chain variable region of D2E7 (D2E7 VL; also shown in SEQ ID NO: 1), alanine-scan mutants of D2E7 VL (LD2E7\*.A1, LD2E7\*.A3, LD2E7\*.A4, LD2E7\*.A5, LD2E7\*.A7 and  
 10 LD2E7\*.A8), the light chain variable region of the D2E7-related antibody 2SD4 (2SD4 VL; also shown in SEQ ID NO: 9) and other D2E7-related light chain variable regions (EP B12, VL10E4, VL100A9, VL100D2, VL10F4, LOE5, VLLOF9, VLL0F10, VLLOG7, VLLOG9, VLLOH1, VLLOH10, VL1B7, VL1C1, VL1C7, VL0.1F4, VL0.1H8, LOE7, LOE7.A and LOE7.T). Figure 1A shows the FR1, CDR1, FR2 and  
 15 CDR2 domains. Figure 1B shows the FR3, CDR3 and FR4 domains. The light chain CDR1 ("CDR L1"), CDR2 ("CDR L2") and CDR3 ("CDR L3") domains are boxed.

Figures 2A and 2B show the amino acid sequences of the heavy chain variable region of D2E7 (D2E7 VH; also shown in SEQ ID NO: 2), alanine-scan mutants of D2E7 VH (HD2E7\*.A1, HD2E7\*.A2, HD2E7\*.A3, HD2E7\*.A4, HD2E7\*.A5,  
 20 HD2E7\*.A6, HD2E7\*.A7, HD2E7\*.A8 and HD2E7\*.A9), the heavy chain variable region of the D2E7-related antibody 2SD4 (2SD4 VH; also shown in SEQ ID NO: 10) and other D2E7-related heavy chain variable regions (VH1B11, VH1D8, VH1A11, VH1B12, VH1-D2, VH1E4, VH1F6, VH1G1, 3C-H2, VH1-D2.N and VH1-D2.Y). Figure 2A shows the FR1, CDR1, FR2 and CDR2 domains. Figure 2B shows the FR3,  
 25 CDR3 and FR4 domains. The heavy chain CDR1 ("CDR H1"), CDR2 ("CDR H2") and CDR3 ("CDR H3") domains are boxed.

Figure 3 is a graph depicting the inhibition of TNF $\alpha$ -induced L929 cytotoxicity by the human anti-hTNF $\alpha$  antibody D2E7, as compared to the murine anti-hTNF $\alpha$  antibody MAK 195.

30 Figure 4 is a graph depicting the inhibition of rhTNF $\alpha$  binding to hTNF $\alpha$  receptors on U-937 cells by the human anti-hTNF $\alpha$  antibody D2E7, as compared to the murine anti-hTNF $\alpha$  antibody MAK 195.

Figure 5 is a graph depicting the inhibition of TNF $\alpha$ -induced ELAM-1 expression on HUVEC by the human anti-hTNF $\alpha$  antibody D2E7, as compared to the  
 35 murine anti-hTNF $\alpha$  antibody MAK 195.

Figure 6 is a bar graph depicting protection from TNF $\alpha$ -induced lethality in



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D-galactosamine-sensitized mice by administration of the human anti-hTNF $\alpha$  antibody D2E7 (black bars), as compared to the murine anti-hTNF $\alpha$  antibody MAK 195 (hatched bars).

Figure 7 shows the nucleotide sequence of the light chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR L1, CDR L2 and CDR L3 regions are underlined.

Figure 8 shows the nucleotide sequence of the heavy chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR H1, CDR H2 and CDR H3 regions are underlined.

Figure 9 is a graph depicting the effect of D2E7 antibody treatment on the mean joint size of Tg197 transgenic mice as a polyarthritis model.

#### **Detailed Description of the Invention**

This invention pertains to isolated human antibodies, or antigen-binding portions thereof, that bind to human TNF $\alpha$  with high affinity, a low off rate and high neutralizing capacity. Various aspects of the invention relate to antibodies and antibody fragments, and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such antibodies and fragments. Methods of using the antibodies of the invention to detect human TNF $\alpha$  or to inhibit human TNF $\alpha$  activity, either *in vitro* or *in vivo*, are also encompassed by the invention.

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNF $\alpha$ " (abbreviated herein as hTNF $\alpha$ , or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNF $\alpha$  is described further in, for example, Pennica, D., *et al.* (1984) *Nature* 312:724-729; Davis, J.M., *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y., *et al.* (1989) *Nature* 338:225-228. The term human TNF $\alpha$  is intended to include recombinant human TNF $\alpha$  (rhTNF $\alpha$ ), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN).

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant

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region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

10       The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, hTNF $\alpha$ ). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')<sub>2</sub> fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123).

35       Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecules, formed by covalent or noncovalent association of the

antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S.M., *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S.M., *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and F(ab')<sub>2</sub> fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further in Section II. below), antibodies isolated from a recombinant combinatorial human antibody library (described further in Section III, below), antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNF $\alpha$  is substantially free of antibodies that specifically bind antigens other than hTNF $\alpha$ ). An isolated antibody that specifically binds hTNF $\alpha$  may, however, have cross-reactivity to other antigens, such as TNF $\alpha$  molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody", as used herein (or an "antibody that neutralized hTNF $\alpha$  activity"), is intended to refer to an antibody whose binding to hTNF $\alpha$  results in inhibition of the biological activity of hTNF $\alpha$ . This inhibition of the biological activity of hTNF $\alpha$  can be assessed by measuring one or more indicators of hTNF $\alpha$  biological activity, such as hTNF $\alpha$ -induced cytotoxicity (either *in vitro* or *in vivo*), hTNF $\alpha$ -induced cellular activation and hTNF $\alpha$  binding to hTNF $\alpha$  receptors. These indicators of hTNF $\alpha$  biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see Example 4). Preferably, the ability of an antibody to neutralize hTNF $\alpha$  activity is assessed by inhibition of hTNF $\alpha$ -induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNF $\alpha$  activity, the ability of an antibody to inhibit hTNF $\alpha$ -induced expression of ELAM-1 on HUVEC, as a measure of hTNF $\alpha$ -induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnson, B., *et al.* (1995) *J. Mol. Recognit.* 8:125-131; and Johnson, B., *et al.* (1991) *Anal. Biochem.* 198:268-277.

The term " $K_{off}$ ", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term " $K_d$ ", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNF $\alpha$ ,

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is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNF $\alpha$ , which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-TNF $\alpha$  antibody contains no other sequences encoding other VH regions that bind antigens other than TNF $\alpha$ .

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

Various aspects of the invention are described in further detail in the following subsections.

35

### I. Human Antibodies that Bind Human TNF $\alpha$

This invention provides isolated human antibodies, or antigen-binding portions thereof, that bind to human TNF $\alpha$  with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNF $\alpha$  antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 and has VL and VH sequences as shown in Figure 1A, 1B and Figure 2A, 2B, respectively (the amino acid sequence of the D2E7 VL region is also shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is also shown in SEQ ID NO: 2). The binding properties of D2E7, as compared to the murine anti-hTNF $\alpha$  MAK 195 mAb that exhibits high affinity and slow dissociation kinetics and another human anti-hTNF $\alpha$  antibody related in sequence to D2E7, 2SD4, are summarized below:

Antibody	$K_{\text{off}}$ $\text{sec}^{-1}$	$k_{\text{on}}$ $\text{M}^{-1} \text{sec}^{-1}$	$K_{\text{d}}$ M	Stoichiometry
D2E7 IgG1	$8.81 \times 10^{-5}$	$1.91 \times 10^5$	$6.09 \times 10^{-10}$	1.2
2SD4 IgG4	$8.4 \times 10^{-3}$	$4.20 \times 10^5$	$2.00 \times 10^{-8}$	0.8
MAK 195 F(ab') <sub>2</sub>	$8.70 \times 10^{-5}$	$1.90 \times 10^5$	$4.60 \times 10^{-10}$	1.4

The D2E7 antibody, and related antibodies, also exhibit a strong capacity to neutralize hTNF $\alpha$  activity, as assessed by several *in vitro* and *in vivo* assays (see Example 4). For example, these antibodies neutralize hTNF $\alpha$ -induced cytotoxicity of L929 cells with IC<sub>50</sub> values in the range of about  $10^{-7}$  M to about  $10^{-10}$  M. D2E7, when expressed as a full-length IgG1 antibody, neutralizes hTNF $\alpha$ -induced cytotoxicity of L929 cells with IC<sub>50</sub> of about  $1.25 \times 10^{-10}$  M. Moreover, the neutralizing capacity of D2E7 is maintained when the antibody is expressed as a Fab, F(ab')<sub>2</sub> or scFv fragment. D2E7 also inhibits TNF $\alpha$ -induced cellular activation, as measured by hTNF $\alpha$ -induced ELAM-1 expression on HUVEC (IC<sub>50</sub> = about  $1.85 \times 10^{-10}$  M), and binding of hTNF $\alpha$  to hTNF $\alpha$  receptors on U-937 cells (IC<sub>50</sub> = about  $1.56 \times 10^{-10}$  M). Regarding the latter, D2E7 inhibits the binding of hTNF $\alpha$  to both the p55 and p75 hTNF $\alpha$  receptors. Furthermore, the antibody inhibits hTNF $\alpha$ -induced lethality *in vivo* in mice (ED<sub>50</sub> = 1-2.5  $\mu\text{g}/\text{mouse}$ ).

Regarding the binding specificity of D2E7, this antibody binds to human TNF $\alpha$  in various forms, including soluble hTNF $\alpha$ , transmembrane hTNF $\alpha$  and hTNF $\alpha$  bound to cellular receptors. D2E7 does not specifically bind to other cytokines, such as lymphotoxin (TNF $\beta$ ), IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IFN $\gamma$  and TGF $\beta$ . However,

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D2E7 does exhibit crossreactivity to tumor necrosis factors from other species. For example, the antibody neutralizes the activity of at least five primate TNF $\alpha$ s (chimpanzee, baboon, marmoset, cynomolgus and rhesus) with approximately equivalent IC<sub>50</sub> values as for neutralization of hTNF $\alpha$  (see Example 4, subsection E).

- 5 D2E7 also neutralizes the activity of mouse TNF $\alpha$ , although approximately 1000-fold less well than human TNF $\alpha$  (see Example 4, subsection E). D2E7 also binds to canine and porcine TNF $\alpha$ .

- In one aspect, the invention pertains to D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and  
10 antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNF $\alpha$  with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNF $\alpha$  with a K<sub>d</sub> of 1 x 10<sup>-8</sup> M or less and a K<sub>off</sub> rate constant of 1 x 10<sup>-3</sup> s<sup>-1</sup> or less, both determined by surface plasmon resonance,  
15 and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an IC<sub>50</sub> of 1 x 10<sup>-7</sup> M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a K<sub>off</sub> of 5 x 10<sup>-4</sup> s<sup>-1</sup> or less, or even more preferably, with a K<sub>off</sub> of 1 x 10<sup>-4</sup> s<sup>-1</sup> or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNF $\alpha$  cytotoxicity in a  
20 standard *in vitro* L929 assay with an IC<sub>50</sub> of 1 x 10<sup>-8</sup> M or less, even more preferably with an IC<sub>50</sub> of 1 x 10<sup>-9</sup> M or less and still more preferably with an IC<sub>50</sub> of 5 x 10<sup>-10</sup> M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof. In another preferred embodiment, the antibody also neutralizes TNF $\alpha$ -induced cellular activation, as assessed using a standard  
25 *in vitro* assay for TNF $\alpha$ -induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC).

- Surface plasmon resonance analysis for determining K<sub>d</sub> and K<sub>off</sub> can be performed as described in Example 1. A standard *in vitro* L929 assay for determining IC<sub>50</sub> values is described in Example 4, subsection A. A standard *in vitro* assay for  
30 TNF $\alpha$ -induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC) is described in Example 4, subsection C. Examples of recombinant human antibodies that meet, or are predicted to meet, the aforementioned kinetic and neutralization criteria include antibodies having the following [VH/VL] pairs, the sequences of which are shown in Figures 1A, 1B, 2A and 2B (see also Examples 2, 3  
35 and 4 for kinetic and neutralization analyses): [D2E7 VH/D2E7 VL]; [HD2E7\*.A1/D2E7 VL], [HD2E7\*.A2/D2E7 VL], [HD2E7\*.A3/D2E7 VL],

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[HD2E7\*.A4/D2E7 VL], [HD2E7\*.A5/D2E7 VL], [HD2E7\*.A6/D2E7 VL],  
 [HD2E7\*.A7/D2E7 VL], [HD2E7\*.A8/D2E7 VL], [HD2E7\*.A9/D2E7 VL], [D2E7  
 VH/LD2E7\*.A1], [D2E7 VH/LD2E7\*.A4], [D2E7 VH/LD2E7\*.A5], [D2E7  
 VH/LD2E7\*.A7], [D2E7 VH/LD2E7\*.A8], [HD2E7\*.A9/LD2E7\*.A1], [VH1-  
 5 D2/LOE7], [VH1-D2.N/LOE7.T], [VH1-D2.Y/LOE7.A], [VH1-D2.N/LOE7.A], [VH1-  
 D2/EP B12] and [3C-H2/LOE7].

It is well known in the art that antibody heavy and light chain CDR3 domains  
 play an important role in the binding specificity/affinity of an antibody for an antigen.  
 Accordingly, in another aspect, the invention pertains to human antibodies that have  
 10 slow dissociation kinetics for association with hTNF $\alpha$  and that have light and heavy  
 chain CDR3 domains that structurally are identical to or related to those of D2E7. As  
 demonstrated in Example 3, position 9 of the D2E7 VL CDR3 can be occupied by Ala  
 or Thr without substantially affecting the  $K_{off}$ . Accordingly, a consensus motif for the  
 D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ  
 15 ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or  
 Asn, without substantially affecting the  $K_{off}$ . Accordingly, a consensus motif for the  
 D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N)  
 (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the  
 D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at  
 20 position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11  
 within the VH CDR3) without substantially affecting the  $K_{off}$ . Still further, the skilled  
 artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3  
 domains to substitutions by alanine, substitution of other amino acids within the CDR3  
 domains may be possible while still retaining the low off rate constant of the antibody,  
 25 in particular substitutions with conservative amino acids. A "conservative amino acid  
 substitution", as used herein, is one in which one amino acid residue is replaced with  
 another amino acid residue having a similar side chain. Families of amino acid residues  
 having similar side chains have been defined in the art, including basic side chains (*e.g.*,  
 lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid),  
 30 uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine,  
 tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine,  
 proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*,  
 threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine,  
 tryptophan, histidine). Preferably, no more than one to five conservative amino acid  
 35 substitutions are made within the D2E7 VL and/or VH CDR3 domains. More  
 preferably, no more than one to three conservative amino acid substitutions are made



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within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNF $\alpha$ . As shown in Example 3, positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNF $\alpha$  and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above).

Accordingly, in another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, with the following characteristics:

10 a) dissociates from human TNF $\alpha$  with a  $K_{off}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 15 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

20 More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $5 \times 10^{-4} \text{ s}^{-1}$  or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{off}$  of  $1 \times 10^{-4} \text{ s}^{-1}$  or less.

In yet another embodiment, the invention provides an isolated human antibody, 25 or an antigen-binding portion thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine 30 substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 35 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably

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are from the  $V_{\kappa}I$  human germline family, more preferably from the A20 human germline  $V_{\kappa}$  gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B. The framework regions for VH preferably are from the  $V_H3$  human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides an isolated human antibody, or an antigen-binding portions thereof, having D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides a recombinant human antibody, or antigen-binding portion thereof, that neutralizes the activity of human  $TNF\alpha$  but not human  $TNF\beta$ . Preferably, antibody, or antigen-binding portion thereof, also neutralizes the activity of chimpanzee  $TNF\alpha$  and at least one additional primate  $TNF\alpha$  selected from the group consisting of baboon  $TNF\alpha$ , marmoset  $TNF\alpha$ , cynomolgus  $TNF\alpha$  and rhesus  $TNF\alpha$ . Preferably, the antibody, or antigen-binding portion thereof, neutralizes the human, chimpanzee and/or additional primate  $TNF\alpha$  in a

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standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-8}$  M or less, more preferably  $1 \times 10^{-9}$  M or less, and even more preferably  $5 \times 10^{-10}$  M or less. In one subembodiment, the antibody also neutralizes the activity of canine TNF $\alpha$ , preferably in a standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-7}$  M or less, more preferably  $1 \times 10^{-8}$  M or less and  
5 even more preferably  $5 \times 10^{-9}$  M or less. In another subembodiment, the antibody also neutralizes the activity of pig TNF $\alpha$ , preferably with an  $IC_{50}$  of  $1 \times 10^{-5}$  M or less, more preferably  $1 \times 10^{-6}$  M or less and even more preferably  $5 \times 10^{-7}$  M or less. In yet another embodiment, the antibody also neutralizes the activity of mouse TNF $\alpha$ , preferably with an  $IC_{50}$  of  $1 \times 10^{-4}$  M or less, more preferably  $1 \times 10^{-5}$  M or less and even more  
10 preferably  $5 \times 10^{-6}$  M or less.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (e.g., another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNF $\alpha$  antibodies described herein,  
15 including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (e.g., a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate  
20 associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, e.g., to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly  
25 reactive groups separated by an appropriate spacer (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (e.g., disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent  
30 detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme  
35 uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and

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diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

5 II. Expression of Antibodies

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin  
10 light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host  
15 cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light  
20 and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of*  
25 *Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V<sub>H</sub> Sequences Reveals about Fifty Groups of V<sub>H</sub> Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V<sub>K</sub> Segments Reveals a Strong Bias in their Usage" *Eur.*  
30 *J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V<sub>H</sub>3 family of human germline V<sub>H</sub> genes is amplified by standard PCR. Most preferably, the DP-31 V<sub>H</sub> germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of  
35 D2E7, or a D2E7-related antibody, a member of the V<sub>K</sub>1 family of human germline V<sub>L</sub> genes is amplified by standard PCR. Most preferably, the A20 V<sub>L</sub> germline sequence is

amplified. PCR primers suitable for use in amplifying the DP-31 germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be  
5 mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that  
10 the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made.

Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

15 Moreover, it should be noted that if the "germline" sequences obtained by PCR amplification encode amino acid differences in the framework regions from the true germline configuration (*i.e.*, differences in the amplified sequence as compared to the true germline sequence, for example as a result of somatic mutation), it may be desirable to change these amino acid differences back to the true germline sequences  
20 (*i.e.*, "backmutation" of framework residues to the germline configuration).

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody  
25 chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA  
30 fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A.,  
35 *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA

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fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another

5 DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*,

10 Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

15 To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence (Gly<sub>4</sub>-Ser)<sub>3</sub>, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl.*  
20 *Acad. Sci. USA* 85:5879-5883; McCafferty *et al.*, *Nature* (1990) 348:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended  
25 to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be  
30 inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression  
35 vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length

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antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

10 In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.*

20 Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes

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include the dihydrofolate reductase (DHFR) gene (for use in dhfr<sup>-</sup> host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr<sup>-</sup> CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNF $\alpha$ . The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are



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specific for an antigen other than hTNF $\alpha$  by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr- CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

In view of the foregoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of the antibodies and antibody portions of the invention. The nucleotide sequence encoding the D2E7 light chain variable region is shown in Figure 7 and SEQ ID NO: 36. The CDR1 domain of the LCVR encompasses nucleotides 70-102, the CDR2 domain encompasses nucleotides 148-168 and the CDR3 domain encompasses nucleotides 265-291. The nucleotide sequence encoding the D2E7 heavy chain variable region is shown in Figure 8 and SEQ ID NO: 37. The CDR1 domain of the HCVR encompasses nucleotides 91-105, the CDR2 domain encompasses nucleotides 148-198 and the CDR3 domain encompasses nucleotides 295-330. It will be appreciated by the skilled artisan that nucleotide sequences encoding D2E7-related antibodies, or portions thereof (e.g., a CDR domain, such as a CDR3 domain), can be derived from the nucleotide sequences encoding the D2E7 LCVR and HCVR using the genetic code and standard molecular biology techniques.

In one embodiment, the invention provides an isolated nucleic acid encoding a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 (*i.e.*, the D2E7 VL CDR3), or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9. This nucleic acid can encode only the CDR3 region

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or, more preferably, encodes an entire antibody light chain variable region (LCVR). For example, the nucleic acid can encode an LCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1).

5 In another embodiment, the invention provides an isolated nucleic acid encoding a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4 (*i.e.*, the D2E7 VH CDR3), or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12. This nucleic acid can  
10 encode only the CDR3 region or, more preferably, encodes an entire antibody heavy chain variable region (HCVR). For example, the nucleic acid can encode a HCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1).

15 In yet another embodiment, the invention provides isolated nucleic acids encoding a D2E7-related CDR3 domain, *e.g.*, comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO 4, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21,  
20 SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody light chain variable region comprising the amino acid sequence of  
25 SEQ ID NO: 1 (*i.e.*, the D2E7 LCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 36, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 1. The nucleic acid can encode only the LCVR or can also encode an antibody light chain constant region, operatively linked to the LCVR.  
30 In one embodiment, this nucleic acid is in a recombinant expression vector.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 HCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 37, although the skilled artisan will appreciate that  
35 due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 2. The nucleic acid can encode only the HCVR or

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can also encode a heavy chain constant region, operatively linked to the HCVR. For example, the nucleic acid can comprise an IgG1 or IgG4 constant region. In one embodiment, this nucleic acid is in a recombinant expression vector.

The invention also provides recombinant expression vectors encoding both an antibody heavy chain and an antibody light chain. For example, in one embodiment, the invention provides a recombinant expression vector encoding:

a) an antibody light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 LCVR); and

b) an antibody heavy chain having a variable region comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 HCVR).

The invention also provides host cells into which one or more of the recombinant expression vectors of the invention have been introduced. Preferably, the host cell is a mammalian host cell, more preferably the host cell is a CHO cell, an NS0 cell or a COS cell.

Still further the invention provides a method of synthesizing a recombinant human antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant human antibody of the invention is synthesized. The method can further comprise isolating the recombinant human antibody from the culture medium.

### III. Selection of Recombinant Human Antibodies

Recombinant human antibodies of the invention in addition to the D2E7 or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*<sup>TM</sup> phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992)

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- Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982.

In a preferred embodiment, to isolate human antibodies with high affinity and a low off rate constant for hTNF $\alpha$ , a murine anti-hTNF $\alpha$  antibody having high affinity and a low off rate constant for hTNF $\alpha$  (e.g., MAK 195, the hybridoma for which has deposit number ECACC 87 050801) is first used to select human heavy and light chain sequences having similar binding activity toward hTNF $\alpha$ , using the epitope imprinting, or guided selection, methods described in Hoogenboom *et al.*, PCT Publication No. WO 93/06213. The antibody libraries used in this method are preferably scFv libraries prepared and screened as described in McCafferty *et al.*, PCT Publication No. WO 92/01047, McCafferty *et al.*, *Nature* (1990) 348:552-554; and Griffiths *et al.*, (1993) *EMBO J* 12:725-734. The scFv antibody libraries preferably are screened using recombinant human TNF $\alpha$  as the antigen.

Once initial human VL and VH segments are selected, "mix and match" experiments, in which different pairs of the initially selected VL and VH segments are screened for hTNF $\alpha$  binding, are performed to select preferred VL/VH pair combinations. Additionally, to further improve the affinity and/or lower the off rate constant for hTNF $\alpha$  binding, the VL and VH segments of the preferred VL/VH pair(s) can be randomly mutated, preferably within the CDR3 region of VH and/or VL, in a process analogous to the *in vivo* somatic mutation process responsible for affinity maturation of antibodies during a natural immune response. This *in vitro* affinity maturation can be accomplished by amplifying VH and VL regions using PCR primers complimentary to the VH CDR3 or VL CDR3, respectively, which primers have been "spiked" with a random mixture of the four nucleotide bases at certain positions such that the resultant PCR products encode VH and VL segments into which random mutations have been introduced into the VH and/or VL CDR3 regions. These randomly mutated VH and VL segments can be rescreened for binding to hTNF $\alpha$  and sequences that exhibit high affinity and a low off rate for hTNF $\alpha$  binding can be selected.

The amino acid sequences of selected antibody heavy and light chains can be compared to germline heavy and light chain amino acid sequences. In cases where certain framework residues of the selected VL and/or VH chains differ from the germline configuration (e.g., as a result of somatic mutation of the immunoglobulin

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genes used to prepare the phage library), it may be desirable to "backmutate" the altered framework residues of the selected antibodies to the germline configuration (*i.e.*, change the framework amino acid sequences of the selected antibodies so that they are the same as the germline framework amino acid sequences). Such "backmutation" (or  
5 "germlining") of framework residues can be accomplished by standard molecular biology methods for introducing specific mutations (*e.g.*, site-directed mutagenesis; PCR-mediated mutagenesis, and the like).

Following screening and isolation of an anti-hTNF $\alpha$  antibody of the invention from a recombinant immunoglobulin display library, nucleic acid encoding the selected  
10 antibody can be recovered from the display package (*e.g.*, from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques. If desired, the nucleic acid can be further manipulated to create other antibody forms of the invention (*e.g.*, linked to nucleic acid encoding additional immunoglobulin domains, such as additional constant regions). To express a recombinant human antibody isolated  
15 by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into a mammalian host cells, as described in further detail in Section II above.

#### IV. Pharmaceutical Compositions and Pharmaceutical Administration

20 The antibodies and antibody-portions of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody or antibody portion of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and  
25 antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium  
30 chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody or antibody portion.

The compositions of this invention may be in a variety of forms. These include,  
35 for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders,

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liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies. The preferred mode of  
5 administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions  
10 of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed  
15 by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any  
20 additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example,  
25 monostearate salts and gelatin.

The antibodies and antibody-portions of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary  
30 depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen,  
35 polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*,

*Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, an antibody or antibody portion of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

- 5 The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the  
10 invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

- Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention  
15 is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating disorders in which TNF $\alpha$  activity is detrimental. For example, an anti-hTNF $\alpha$  antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface  
20 molecules), one or more cytokines, soluble TNF $\alpha$  receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNF $\alpha$  production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination  
25 therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

- Nonlimiting examples of therapeutic agents for rheumatoid arthritis with which an antibody, or antibody portion, of the invention can be combined include the  
30 following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2 (chimeric anti-TNF $\alpha$  antibody; Centocor); 75 kDTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kDTNFR-IgG  
35 IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see *e.g.*,

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- Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2R $\alpha$ ; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNFR (soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1 $\beta$  converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120);



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gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see e.g., DeLuca et al. (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); and azaribine.

Nonlimiting examples of therapeutic agents for inflammatory bowel disease with which an antibody, or antibody portion, of the invention can be combined include the following: budesonide; epidermal growth factor; corticosteroids; cyclosporin, sulfasalazine; aminosalicylates; 6-mercaptopurine; azathioprine; metronidazole; lipoxygenase inhibitors; mesalamine; olsalazine; balsalazide; antioxidants; thromboxane inhibitors; IL-1 receptor antagonists; anti-IL-1 $\beta$  monoclonal antibodies; anti-IL-6 monoclonal antibodies; growth factors; elastase inhibitors; pyridinyl-imidazole compounds; CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2 (chimeric anti-TNF $\alpha$  antibody; Centocor); 75 kDTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kDTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); interleukin-10 (SCH 52000; Schering Plough); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); interleukin-11; glucuronide- or dextran-conjugated prodrugs of prednisolone, dexamethasone or budesonide; ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); slow-release mesalazine; methotrexate; antagonists of Platelet Activating Factor (PAF); ciprofloxacin; and lignocaine.

Nonlimiting examples of therapeutic agents for multiple sclerosis with which an antibody, or antibody portion, of the invention can be combined include the following: corticosteroids; prednisolone; methylprednisolone; azathioprine; cyclophosphamide; cyclosporine; methotrexate; 4-aminopyridine; tizanidine; interferon- $\beta$ 1a (Avonex<sup>TM</sup>; Biogen); interferon- $\beta$ 1b (Betaseron<sup>TM</sup>; Chiron/Berlex); Copolymer 1 (Cop-1; Copaxone<sup>TM</sup>; Teva Pharmaceutical Industries, Inc.); hyperbaric oxygen; intravenous

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immunoglobulin; clabribine; CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2 (chimeric anti-TNF $\alpha$  antibody; Centocor); 75 kDTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kDTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IL-10; IL-4; and IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies).

Nonlimiting examples of therapeutic agents for sepsis with which an antibody, or antibody portion, of the invention can be combined include the following: hypertonic saline solutions; antibiotics; intravenous gamma globulin; continuous hemofiltration; carbapenems (*e.g.*, meropenem); antagonists of cytokines such as TNF $\alpha$ , IL-1 $\beta$ , IL-6 and/or IL-8; CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2 (chimeric anti-TNF $\alpha$  antibody; Centocor); 75 kDTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kDTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); SK&F 107647 (low molecular peptide; SmithKline Beecham); tetravalent guanyldiazide CNI-1493 (Picower Institute); Tissue Factor Pathway Inhibitor (TFPI; Chiron); PHP (chemically modified hemoglobin; APEX Bioscience); iron chelators and chelates, including diethylenetriamine pentaacetic acid - iron (III) complex (DTPA iron (III); Molichem Medicines); lisofylline (synthetic small molecule methylxanthine; Cell Therapeutics, Inc.); PGG-Glucan (aqueous soluble  $\beta$ 1,3glucan; Alpha-Beta Technology); apolipoprotein A-1 reconstituted with lipids; chiral hydroxamic acids (synthetic antibacterials that inhibit lipid A biosynthesis); anti-endotoxin antibodies; E5531 (synthetic lipid A antagonist; Eisai America, Inc.); rBPI<sub>21</sub> (recombinant N-terminal fragment of human Bactericidal/Permeability-Increasing Protein); and Synthetic Anti-Endotoxin Peptides (SAEP; Biosynth Research Laboratories);

Nonlimiting examples of therapeutic agents for adult respiratory distress syndrome (ARDS) with which an antibody, or antibody portion, of the invention can be combined include the following: anti-IL-8 antibodies; surfactant replacement therapy; CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2 (chimeric anti-TNF $\alpha$  antibody; Centocor); 75 kDTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); and 55 kDTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche).

The use of the antibodies, or antibody portions, of the invention in combination with other therapeutic agents is discussed further in subsection IV.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody  
5 portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody or antibody portion may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the  
10 individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at  
15 an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be  
20 proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound  
25 calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the  
30 treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 0.1-20 mg/kg, more preferably 1-10 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any  
35 particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or

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supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

5 IV. Uses of the Antibodies of the Invention

Given their ability to bind to hTNF $\alpha$ , the anti-hTNF $\alpha$  antibodies, or portions thereof, of the invention can be used to detect hTNF $\alpha$  (e.g., in a biological sample, such as serum or plasma), using a conventional immunoassay, such as an enzyme linked immunosorbent assays (ELISA), an radioimmunoassay (RIA) or tissue  
10 immunohistochemistry. The invention provides a method for detecting hTNF $\alpha$  in a biological sample comprising contacting a biological sample with an antibody, or antibody portion, of the invention and detecting either the antibody (or antibody portion) bound to hTNF $\alpha$  or unbound antibody (or antibody portion), to thereby detect hTNF $\alpha$  in the biological sample. The antibody is directly or indirectly labeled with a detectable  
15 substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and  
20 avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

Alternative to labeling the antibody, hTNF $\alpha$  can be assayed in biological fluids  
25 by a competition immunoassay utilizing rhTNF $\alpha$  standards labeled with a detectable substance and an unlabeled anti-hTNF $\alpha$  antibody. In this assay, the biological sample, the labeled rhTNF $\alpha$  standards and the anti-hTNF $\alpha$  antibody are combined and the amount of labeled rhTNF $\alpha$  standard bound to the unlabeled antibody is determined. The amount of hTNF $\alpha$  in the biological sample is inversely proportional to the amount of  
30 labeled rhTNF $\alpha$  standard bound to the anti-hTNF $\alpha$  antibody.

A D2E7 antibody of the invention can also be used to detect TNF $\alpha$ s from species other than humans, in particular TNF $\alpha$ s from primates (e.g., chimpanzee, baboon, marmoset, cynomolgus and rhesus), pig and mouse, since D2E7 can bind to each of these TNF $\alpha$ s (discussed further in Example 4, subsection E).

35 The antibodies and antibody portions of the invention are capable of neutralizing hTNF $\alpha$  activity both *in vitro* and *in vivo* (see Example 4). Moreover, at least some of

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the antibodies of the invention, such as D2E7, can neutralize TNF $\alpha$  activity from other species. Accordingly, the antibodies and antibody portions of the invention can be used to inhibit TNF $\alpha$  activity, *e.g.*, in a cell culture containing hTNF $\alpha$ , in human subjects or in other mammalian subjects having TNF $\alpha$ s with which an antibody of the invention

5 cross-reacts (*e.g.* chimpanzee, baboon, marmoset, cynomolgus and rhesus, pig or mouse). In one embodiment, the invention provides a method for inhibiting TNF $\alpha$  activity comprising contacting TNF $\alpha$  with an antibody or antibody portion of the invention such that TNF $\alpha$  activity is inhibited. Preferably, the TNF $\alpha$  is human TNF $\alpha$ . For example, in a cell culture containing, or suspected of containing hTNF $\alpha$ , an

10 antibody or antibody portion of the invention can be added to the culture medium to inhibit hTNF $\alpha$  activity in the culture.

In another embodiment, the invention provides a method for inhibiting TNF $\alpha$  activity in a subject suffering from a disorder in which TNF $\alpha$  activity is detrimental. TNF $\alpha$  has been implicated in the pathophysiology of a wide variety of disorders (see

15 *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A.). The invention provides methods for TNF $\alpha$  activity in a subject suffering from such a disorder, which method comprises administering to the subject an antibody or antibody portion of the invention such that TNF $\alpha$  activity in the subject is inhibited. Preferably,

20 the TNF $\alpha$  is human TNF $\alpha$  and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNF $\alpha$  with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNF $\alpha$  (*e.g.*, by administration of hTNF $\alpha$  or by expression of an hTNF $\alpha$  transgene). An antibody of the invention can be administered to a human subject for therapeutic

25 purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNF $\alpha$  with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages

30 and time courses of administration).

As used herein, the term "a disorder in which TNF $\alpha$  activity is detrimental" is intended to include diseases and other disorders in which the presence of TNF $\alpha$  in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a

35 worsening of the disorder. Accordingly, a disorder in which TNF $\alpha$  activity is detrimental is a disorder in which inhibition of TNF $\alpha$  activity is expected to alleviate the

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symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNF $\alpha$  in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNF $\alpha$  in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an  
5 anti-TNF $\alpha$  antibody as described above. There are numerous examples of disorders in which TNF $\alpha$  activity is detrimental. The use of the antibodies and antibody portions of the invention in the treatment of specific disorders is discussed further below:

#### A. Sepsis

10 Tumor necrosis factor has an established role in the pathophysiology of sepsis, with biological effects that include hypotension, myocardial suppression, vascular leakage syndrome, organ necrosis, stimulation of the release of toxic secondary mediators and activation of the clotting cascade (see *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent  
15 Publication No. 260 610 B1 by Moeller, A.; Tracey, K.J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503; Russell, D and Thompson, R.C. (1993) *Curr. Opin. Biotech.* 4:714-721). Accordingly, the human antibodies, and antibody portions, of the invention can be used to treat sepsis in any of its clinical settings, including septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome.

20 Furthermore, to treat sepsis, an anti-hTNF $\alpha$  antibody, or antibody portion, of the invention can be coadministered with one or more additional therapeutic agents that may further alleviate sepsis, such as an interleukin-1 inhibitor (such as those described in PCT Publication Nos. WO 92/16221 and WO 92/17583), the cytokine interleukin-6 (see  
25 *e.g.*, PCT Publication No. WO 93/11793) or an antagonist of platelet activating factor (see *e.g.*, European Patent Application Publication No. EP 374 510). Other combination therapies for the treatment of sepsis are discussed further in subsection III.

Additionally, in a preferred embodiment, an anti-TNF $\alpha$  antibody or antibody portion of the invention is administered to a human subject within a subgroup of sepsis patients having a serum or plasma concentration of IL-6 above 500 pg/ml. and more  
30 preferably 1000 pg/ml, at the time of treatment (see PCT Publication No. WO 95/20978 by Daum, L., *et al.*).

#### B. Autoimmune Diseases

Tumor necrosis factor has been implicated in playing a role in the  
35 pathophysiology of a variety of autoimmune diseases. For example, TNF $\alpha$  has been implicated in activating tissue inflammation and causing joint destruction in rheumatoid

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arthritis (see *e.g.*, Moeller, A., *et al.* (1990) *Cytokine* 2:162-169; U.S. Patent No. 5,231,024 to Moeller *et al.*; European Patent Publication No. 260 610 B1 by Moeller, A.; Tracey and Cerami, *supra*; Arend, W.P. and Dayer, J-M. (1995) *Arth. Rheum.* 38:151-160; Fava, R.A., *et al.* (1993) *Clin. Exp. Immunol.* 94:261-266). TNF $\alpha$  also has  
5 been implicated in promoting the death of islet cells and in mediating insulin resistance in diabetes (see *e.g.*, Tracey and Cerami, *supra*; PCT Publication No. WO 94/08609). TNF $\alpha$  also has been implicated in mediating cytotoxicity to oligodendrocytes and induction of inflammatory plaques in multiple sclerosis (see *e.g.*, Tracey and Cerami, *supra*). Chimeric and humanized murine anti-hTNF $\alpha$  antibodies have undergone  
10 clinical testing for treatment of rheumatoid arthritis (see *e.g.*, Elliott, M.J., *et al.* (1994) *Lancet* 344:1125-1127; Elliot, M.J., *et al.* (1994) *Lancet* 344:1105-1110; Rankin, E.C., *et al.* (1995) *Br. J. Rheumatol.* 34:334-342).

The human antibodies, and antibody portions of the invention can be used to treat autoimmune diseases, in particular those associated with inflammation, including  
15 rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome. Typically, the antibody, or antibody portion, is administered systemically, although for certain disorders, local administration of the antibody or antibody portion at a site of inflammation may be beneficial (*e.g.*, local administration in the joints in rheumatoid  
20 arthritis or topical application to diabetic ulcers, alone or in combination with a cyclohexane-ylidene derivative as described in PCT Publication No. WO 93/19751). An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of autoimmune diseases, as discussed further in subsection III.

25

### C. Infectious Diseases

Tumor necrosis factor has been implicated in mediating biological effects observed in a variety of infectious diseases. For example, TNF $\alpha$  has been implicated in mediating brain inflammation and capillary thrombosis and infarction in malaria. TNF $\alpha$   
30 also has been implicated in mediating brain inflammation, inducing breakdown of the blood-brain barrier, triggering septic shock syndrome and activating venous infarction in meningitis. TNF $\alpha$  also has been implicated in inducing cachexia, stimulating viral proliferation and mediating central nervous system injury in acquired immune deficiency syndrome (AIDS). Accordingly, the antibodies, and antibody portions, of the  
35 invention, can be used in the treatment of infectious diseases, including bacterial meningitis (see *e.g.*, European Patent Application Publication No. EP 585 705), cerebral

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malaria, AIDS and AIDS-related complex (ARC) (see *e.g.*, European Patent Application Publication No. EP 230 574), as well as cytomegalovirus infection secondary to transplantation (see *e.g.*, Fietze, E., *et al.* (1994) *Transplantation* 58:675-680). The antibodies, and antibody portions, of the invention, also can be used to alleviate  
5 symptoms associated with infectious diseases, including fever and myalgias due to infection (such as influenza) and cachexia secondary to infection (*e.g.*, secondary to AIDS or ARC).

#### D. Transplantation

10 Tumor necrosis factor has been implicated as a key mediator of allograft rejection and graft versus host disease (GVHD) and in mediating an adverse reaction that has been observed when the rat antibody OKT3, directed against the T cell receptor CD3 complex, is used to inhibit rejection of renal transplants (see *e.g.*, Eason, J.D., *et al.* (1995) *Transplantation* 59:300-305; Suthanthiran, M. and Strom, T.B. (1994) *New Engl.*  
15 *J. Med.* 331:365-375). Accordingly, the antibodies, and antibody portions, of the invention, can be used to inhibit transplant rejection, including rejections of allografts and xenografts and to inhibit GVHD. Although the antibody or antibody portion may be used alone, more preferably it is used in combination with one or more other agents that inhibit the immune response against the allograft or inhibit GVHD. For example, in one  
20 embodiment, an antibody or antibody portion of the invention is used in combination with OKT3 to inhibit OKT3-induced reactions. In another embodiment, an antibody or antibody portion of the invention is used in combination with one or more antibodies directed at other targets involved in regulating immune responses, such as the cell surface molecules CD25 (interleukin-2 receptor- $\alpha$ ), CD11a (LFA-1), CD54 (ICAM-1),  
25 CD4, CD45, CD28/CTLA4, CD80 (B7-1) and/or CD86 (B7-2). In yet another embodiment, an antibody or antibody portion of the invention is used in combination with one or more general immunosuppressive agents, such as cyclosporin A or FK506.

#### E. Malignancy

30 Tumor necrosis factor has been implicated in inducing cachexia, stimulating tumor growth, enhancing metastatic potential and mediating cytotoxicity in malignancies. Accordingly, the antibodies, and antibody portions, of the invention, can be used in the treatment of malignancies, to inhibit tumor growth or metastasis and/or to alleviate cachexia secondary to malignancy. The antibody, or antibody portion, may be  
35 administered systemically or locally to the tumor site.



#### F. *Pulmonary Disorders*

Tumor necrosis factor has been implicated in the pathophysiology of adult respiratory distress syndrome (ARDS), including stimulating leukocyte-endothelial activation, directing cytotoxicity to pneumocytes and inducing vascular leakage syndrome. Accordingly, the antibodies, and antibody portions, of the invention, can be used to treat various pulmonary disorders, including adult respiratory distress syndrome (see *e.g.*, PCT Publication No. WO 91/04054), shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis and silicosis. The antibody, or antibody portion, may be administered systemically or locally to the lung surface, for example as an aerosol. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of pulmonary disorders, as discussed further in subsection III.

#### G. *Intestinal Disorders*

Tumor necrosis factor has been implicated in the pathophysiology of inflammatory bowel disorders (see *e.g.*, Tracy, K.J., *et al.* (1986) *Science* 234:470-474; Sun, X-M., *et al.* (1988) *J. Clin. Invest.* 81:1328-1331; MacDonald, T.T., *et al.* (1990) *Clin. Exp. Immunol.* 81:301-305). Chimeric murine anti-hTNF $\alpha$  antibodies have undergone clinical testing for treatment of Crohn's disease (van Dullemen, H.M., *et al.* (1995) *Gastroenterology* 109:129-135). The human antibodies, and antibody portions, of the invention, also can be used to treat intestinal disorders, such as idiopathic inflammatory bowel disease, which includes two syndromes. Crohn's disease and ulcerative colitis. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of intestinal disorders, as discussed further in subsection III.

#### H. *Cardiac Disorders*

The antibodies, and antibody portions, of the invention, also can be used to treat various cardiac disorders, including ischemia of the heart (see *e.g.*, European Patent Application Publication No. EP 453 898) and heart insufficiency (weakness of the heart muscle)(see *e.g.*, PCT Publication No. WO 94/20139).

#### I. *Others*

The antibodies, and antibody portions, of the invention, also can be used to treat various other disorders in which TNF $\alpha$  activity is detrimental. Examples of other diseases and disorders in which TNF $\alpha$  activity has been implicated in the

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pathophysiology, and thus which can be treated using an antibody, or antibody portion, of the invention. include inflammatory bone disorders and bone resorption disease (see *e.g.*, Bertolini, D.R., *et al.* (1986) *Nature* 319:516-518; Konig, A., *et al.* (1988) *J. Bone Miner. Res.* 3:621-627; Lerner, U.H. and Ohlin, A. (1993) *J. Bone Miner. Res.* 8:147-155; and Shankar, G. and Stern, P.H. (1993) *Bone* 14:871-876), hepatitis, including alcoholic hepatitis (see *e.g.*, McClain, C.J. and Cohen, D.A. (1989) *Hepatology* 9:349-351; Feiver, M.E., *et al.* (1990) *Alcohol. Clin. Exp. Res.* 14:255-259; and Hansen, J., *et al.* (1994) *Hepatology* 20:461-474), viral hepatitis (Sheron, N., *et al.* (1991) *J. Hepatol.* 12:241-245; and Hussain, M.J., *et al.* (1994) *J. Clin. Pathol.* 47:1112-1115), and fulminant hepatitis; coagulation disturbances (see *e.g.*, van der Poll, T., *et al.* (1990) *N. Engl. J. Med.* 322:1622-1627; and van der Poll, T., *et al.* (1991) *Prog. Clin. Biol. Res.* 367:55-60), burns (see *e.g.*, Giroir, B.P., *et al.* (1994) *Am. J. Physiol.* 267:H118-124; and Liu, X.S., *et al.* (1994) *Burns* 20:40-44), reperfusion injury (see *e.g.*, Scales, W.E., *et al.* (1994) *Am. J. Physiol.* 267:G1122-1127; Serrick, C., *et al.* (1994) *Transplantation* 58:1158-1162; and Yao, Y.M., *et al.* (1995) *Resuscitation* 29:157-168), keloid formation (see *e.g.*, McCauley, R.L., *et al.* (1992) *J. Clin. Immunol.* 12:300-308), scar tissue formation; pyrexia; periodontal disease; obesity and radiation toxicity.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

#### **EXAMPLE 1:            Kinetic Analysis of Binding of Human Antibodies to hTNF $\alpha$**

Real-time binding interactions between ligand (biotinylated recombinant human TNF $\alpha$  (rhTNF $\alpha$ ) immobilized on a biosensor matrix) and analyte (antibodies in solution) were measured by surface plasmon resonance (SPR) using the BIAcore system (Pharmacia Biosensor, Piscataway, NJ). The system utilizes the optical properties of SPR to detect alterations in protein concentrations within a dextran biosensor matrix. Proteins are covalently bound to the dextran matrix at known concentrations. Antibodies are injected through the dextran matrix and specific binding between injected antibodies and immobilized ligand results in an increased matrix protein concentration and resultant change in the SPR signal. These changes in SPR signal are recorded as resonance units (RU) and are displayed with respect to time along the y-axis of a sensorgram.

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To facilitate immobilization of biotinylated rhTNF $\alpha$  on the biosensor matrix, streptavidin is covalently linked via free amine groups to the dextran matrix by first activating carboxyl groups on the matrix with 100 mM N-hydroxysuccinimide (NHS) and 400 mM N-ethyl-N'-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC).

- 5 Next, streptavidin is injected across the activated matrix. Thirty-five microliters of streptavidin (25  $\mu$ g/ml), diluted in sodium acetate, pH 4.5, is injected across the activated biosensor and free amines on the protein are bound directly to the activated carboxyl groups. Unreacted matrix EDC-esters are deactivated by an injection of 1 M ethanolamine. Streptavidin-coupled biosensor chips also are commercially available
- 10 (Pharmacia BR-1000-16, Pharmacia Biosensor, Piscataway, NJ).

- Biotinylated rhTNF $\alpha$  was prepared by first dissolving 5.0 mg of biotin (D-biotinyl- $\epsilon$ -aminocaproic acid N-hydroxysuccinimide ester; Boehringer Mannheim Cat. No. 1008 960) in 500  $\mu$ l dimethylsulfoxide to make a 10 mg/ml solution. Ten microliters of biotin was added per ml of rhTNF $\alpha$  (at 2.65 mg/ml) for a 2:1 molar ratio
- 15 of biotin to rhTNF $\alpha$ . The reaction was mixed gently and incubated for two hours at room temperature in the dark. A PD-10 column, Sephadex G-25M (Pharmacia Catalog No. 17-0851-01) was equilibrated with 25 ml of cold PBS and loaded with 2 ml of rhTNF $\alpha$ -biotin per column. The column was eluted with 10 x 1 ml cold PBS. Fractions were collected and read at OD280 (1.0 OD = 1.25 mg/ml). The appropriate fractions
- 20 were pooled and stored at -80° C until use. Biotinylated rhTNF $\alpha$  also is commercially available (R & D Systems Catalog No. FTA00, Minneapolis, MN).

- Biotinylated rhTNF $\alpha$  to be immobilized on the matrix via streptavidin was diluted in PBS running buffer (Gibco Cat. No. 14190-144, Gibco BRL, Grand Island, NY) supplemented with 0.05% (BIAcore) surfactant P20 (Pharmacia BR-1000-54,
- 25 Pharmacia Biosensor, Piscataway, NJ). To determine the capacity of rhTNF $\alpha$ -specific antibodies to bind immobilized rhTNF $\alpha$ , a binding assay was conducted as follows. Aliquots of biotinylated rhTNF $\alpha$  (25 nM; 10  $\mu$ l aliquots) were injected through the streptavidin-coupled dextran matrix at a flow rate of 5  $\mu$ l/min. Before injection of the protein and immediately afterward, PBS buffer alone flowed through each flow cell.
- 30 The net difference in signal between baseline and approximately 30 sec. after completion of biotinylated rhTNF $\alpha$  injection was taken to represent the binding value (approximately 500 RU). Direct rhTNF $\alpha$ -specific antibody binding to immobilized biotinylated rhTNF $\alpha$  was measured. Antibodies (20  $\mu$ g/ml) were diluted in PBS running buffer and 25  $\mu$ l aliquots were injected through the immobilized protein
- 35 matrices at a flow rate of 5  $\mu$ l/min. Prior to injection of antibody, and immediately afterwards, PBS buffer alone flowed through each flow cell. The net difference in

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- baseline signal and signal after completion of antibody injection was taken to represent the binding value of the particular sample. Biosensor matrices were regenerated using 100 mM HCl before injection of the next sample. To determine the off rate ( $K_{off}$ ), on rate ( $K_{on}$ ), association rate ( $K_a$ ) and dissociation rate ( $K_d$ ) constants, BIAcore kinetic evaluation software (version 2.1) was used.

Representative results of D2E7 (IgG4 full-length antibody) binding to biotinylated rhTNF $\alpha$ , as compared to the mouse mAb MAK 195 (F(ab')<sub>2</sub> fragment), are shown below in Table 1.

10 Table 1: Binding of D2E7 IgG4 or MAK 195 to Biotinylated rhTNF $\alpha$

Antibody	[Ab], nM	rhTNF $\alpha$ , bound, RUs	Ab, bound, RUs	rhTNF $\alpha$ /Ab	$K_{off}$ , sec <sup>-1</sup> , (Avg)
D2E7	267	373	1215	1.14	$8.45 \times 10^{-5}$
	133	420	1569	1.30	$5.42 \times 10^{-5}$
	67	434	1633	1.31	$4.75 \times 10^{-5}$
	33	450	1532	1.19	$4.46 \times 10^{-5}$
	17	460	1296	0.98	$3.47 \times 10^{-5}$
	8	486	936	0.67	$2.63 \times 10^{-5}$
	4	489	536	0.38	$2.17 \times 10^{-5}$
	2	470	244	0.18	$3.68 \times 10^{-5}$
					$(4.38 \times 10^{-5})$
MAK 195	400	375	881	1.20	$5.38 \times 10^{-5}$
	200	400	1080	1.38	$4.54 \times 10^{-5}$
	100	419	1141	1.39	$3.54 \times 10^{-5}$
	50	427	1106	1.32	$3.67 \times 10^{-5}$
	25	446	957	1.09	$4.41 \times 10^{-5}$
	13	464	708	0.78	$3.66 \times 10^{-5}$
	6	474	433	0.47	$7.37 \times 10^{-5}$
	3	451	231	0.26	$6.95 \times 10^{-5}$
					$(4.94 \times 10^{-5})$

- In a second series of experiments, the molecular kinetic interactions between an IgG1 full-length form of D2E7 and biotinylated rhTNF was quantitatively analyzed using BIAcore technology, as described above, and kinetic rate constants were derived, summarized below in Tables 2, 3 and 4.

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Table 2: Apparent dissociation rate constants of the interaction between D2E7 and biotinylated rhTNF

<u>Experiment</u>	$K_d (s^{-1})$
1	$9.58 \times 10^{-5}$
2	$9.26 \times 10^{-5}$
3	$7.60 \times 10^{-5}$
Average	$8.81 \pm 1.06 \times 10^{-5}$

Table 3: Apparent association rate constants of the interaction between D2E7 and biotinylated rhTNF

<u>Experiment</u>	$K_a (M^{-1} s^{-1})$
1	$1.33 \times 10^5$
2	$1.05 \times 10^5$
3	$3.36 \times 10^5$
Average	$1.91 \pm 1.26 \times 10^5$

Table 4: Apparent kinetic rate and affinity constants of D2E7 and biotinylated rhTNF

<u>Experiment</u>	$K_a (M^{-1} s^{-1})$	$K_d (s^{-1})$	$K_d (M)$
1	$1.33 \times 10^5$	$9.58 \times 10^{-5}$	$7.20 \times 10^{-10}$
2	$1.05 \times 10^5$	$9.26 \times 10^{-5}$	$8.82 \times 10^{-10}$
3	$3.36 \times 10^5$	$7.60 \times 10^{-5}$	$2.26 \times 10^{-10}$
Average	$1.91 \pm 1.26 \times 10^5$	$8.81 \pm 1.06 \times 10^{-5}$	$6.09 \pm 3.42 \times 10^{-10}$

Dissociation and association rate constants were calculated by analyzing the dissociation and association regions of the sensorgrams by BIA analysis software. Conventional chemical reaction kinetics were assumed for the interaction between D2E7 and biotinylated rhTNF molecule: a zero order dissociation and first order association kinetics. For the sake of analysis, interaction only between one arm of the bivalent D2E7 antibody and one unit of the trimeric biotinylated rhTNF was considered in choosing molecular models for the analysis of the kinetic data. Three independent experiments were performed and the results were analyzed separately. The average apparent dissociation rate constant ( $k_d$ ) of the interaction between D2E7 and biotinylated rhTNF was  $8.81 \pm 1.06 \times 10^{-5} s^{-1}$ , and the average apparent association rate constant,  $k_a$  was  $1.91 \pm 1.26 \times 10^5 M^{-1} s^{-1}$ . The apparent intrinsic dissociation constant ( $K_d$ ) was then calculated by the formula:  $K_d = k_d / k_a$ . Thus, the mean  $K_d$  of D2E7 antibody for rhTNF

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derived from kinetic parameters was  $6.09 \pm 3.42 \times 10^{-10}$  M. Minor differences in the kinetic values for the IgG1 form of D2E7 (presented in Tables 2, 3 and 4) and the IgG4 form of D2E7 (presented in Table 1 and in Examples 2 and 3) are not thought to be true differences resulting from the presence of either an IgG1 or an IgG4 constant regions but rather are thought to be attributable to more accurate antibody concentration measurements used for the IgG1 kinetic analysis. Accordingly, the kinetic values for the IgG1 form of D2E7 presented herein are thought to be the most accurate kinetic parameters for the D2E7 antibody.

## 10 **EXAMPLE 2: Alanine Scanning Mutagenesis of D2E7 CDR3 Domains**

A series of single alanine mutations were introduced by standard methods along the CDR3 domain of the D2E7 VL and the D2E7 VH regions. The light chain mutations are illustrated in Figure 1B (LD2E7\*.A1, LD2E7\*.A3, LD2E7\*.A4, LD2E7\*.A5, LD2E7\*.A7 and LD2E7\*.A8, having an alanine mutation at position 1, 3, 4, 5, 7 or 8, respectively, of the D2E7 VL CDR3 domain). The heavy chain mutations are illustrated in Figure 2B (HD2E7\*.A1, HD2E7\*.A2, HD2E7\*.A3, HD2E7\*.A4, HD2E7\*.A5, HD2E7\*.A6, HD2E7\*.A7, HD2E7\*.A8 and HD2E7\*.A9, having an alanine mutation at position 2, 3, 4, 5, 6, 8, 9, 10 or 11, respectively, of the D2E7 VH CDR3 domain). The kinetics of rhTNF $\alpha$  interaction with an antibody composed of wild-type D2E7 VL and VH was compared to that of antibodies composed of 1) a wild-type D2E7 VL paired with an alanine-substituted D2E7 VH; 2) a wild-type D2E7 VH paired with an alanine-substituted D2E7 VL; or 3) an alanine-substituted D2E7 VL paired with an alanine-substituted D2E7 VH. All antibodies were tested as full-length, IgG4 molecules.

Kinetics of interaction of antibodies with rhTNF $\alpha$  was determined by surface plasmon resonance as described in Example 1. The  $K_{off}$  rates for the different VH/VL pairs are summarized below in Table 5:

30 **Table 5: Binding of D2E7 Alanine-Scan Mutants to Biotinylated rhTNF $\alpha$**

VH	VL	$K_{off} (sec^{-1})$
D2E7 VH	D2E7 VL	$9.65 \times 10^{-5}$
HD2E7*.A1	D2E7 VL	$1.4 \times 10^{-4}$
HD2E7*.A2	D2E7 VL	$4.6 \times 10^{-4}$
HD2E7*.A3	D2E7 VL	$8.15 \times 10^{-4}$
HD2E7*.A4	D2E7 VL	$1.8 \times 10^{-4}$
HD2E7*.A5	D2E7 VL	$2.35 \times 10^{-4}$

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HD2E7*.A6	D2E7 VL	$2.9 \times 10^{-4}$
HD2E7*.A7	D2E7 VL	$1.0 \times 10^{-4}$
HD2E7*.A8	D2E7 VL	$3.1 \times 10^{-4}$
HD2E7*.A9	D2E7 VL	$8.1 \times 10^{-4}$
D2E7 VH	LD2E7*.A1	$6.6 \times 10^{-5}$
D2E7 VH	LD2E7*.A3	NOT DETECTABLE
D2E7 VH	LD2E7*.A4	$1.75 \times 10^{-4}$
D2E7 VH	LD2E7*.A5	$1.8 \times 10^{-4}$
D2E7 VH	LD2E7*.A7	$1.4 \times 10^{-4}$
D2E7 VH	LD2E7*.A8	$3.65 \times 10^{-4}$
HD2E7*.A9	LD2E7*.A1	$1.05 \times 10^{-4}$

These results demonstrate that the majority of positions of the CDR3 domains of the D2E7 VL region and VH region are amenable to substitution with a single alanine residue. Substitution of a single alanine at position 1, 4, 5, or 7 of the D2E7 VL CDR3 domain or at position 2, 5, 6, 8, 9 or 10 of the D2E7 VH CDR3 domain does not significantly affect the off rate of hTNF $\alpha$  binding as compared to the wild-type parental D2E7 antibody. Substitution of alanine at position 8 of the D2E7 VL CDR3 or at position 3 of the D2E7 VH CDR3 gives a 4-fold faster  $K_{off}$  and an alanine substitution at position 4 or 11 of D2E7 VH CDR3 gives an 8-fold faster  $K_{off}$ , indicating that these positions are more critical for binding to hTNF $\alpha$ . However, a single alanine substitution at position 1, 4, 5, 7 or 8 of the D2E7 VL CDR3 domain or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 of the D2E7 VH CDR3 domain still results in an anti-hTNF $\alpha$  antibody having a  $K_{off}$  of  $1 \times 10^{-3} \text{ sec}^{-1}$  or less.

### 15 **EXAMPLE 3: Binding Analysis of D2E7-Related Antibodies**

A series of antibodies related in sequence to D2E7 were analyzed for their binding to rhTNF $\alpha$ , as compared to D2E7, by surface plasmon resonance as described in Example 1. The amino acid sequences of the VL regions tested are shown in Figures 1A and 1B. The amino acid sequences of the VH regions tested are shown in Figures 2A and 2B. The  $K_{off}$  rates for various VH/VL pairs (in the indicated format, either as a full-length IgG1 or IgG4 antibody or as a scFv) are summarized below in Table 6:

Table 6: Binding of D2E7-Related Antibodies to Biotinylated rhTNF $\alpha$

VH	VL	Format	$K_{off} (\text{sec}^{-1})$
D2E7 VH	D2E7 VL	IgG1/IgG4	$9.65 \times 10^{-5}$

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VH1-D2	LOE7	IgG1/IgG4	$7.7 \times 10^{-5}$
VH1-D2	LOE7	scFv	$4.6 \times 10^{-4}$
VH1-D2.N	LOE7.T	IgG4	$2.1 \times 10^{-5}$
VH1-D2.Y	LOE7.A	IgG4	$2.7 \times 10^{-5}$
VH1-D2.N	LOE7.A	IgG4	$3.2 \times 10^{-5}$
VH1-D2	EP B12	scFv	$8.0 \times 10^{-4}$
VH1-D2	2SD4 VL	scFv	$1.94 \times 10^{-3}$
3C-H2	LOE7	scFv	$1.5 \times 10^{-3}$
2SD4 VH	LOE7	scFv	$6.07 \times 10^{-3}$
2SD4 VH	2SD4 VL	scFv	$1.37 \times 10^{-2}$
VH1A11	2SD4 VL	scFv	$1.34 \times 10^{-2}$
VH1B12	2SD4 VL	scFv	$1.01 \times 10^{-2}$
VH1B11	2SD4 VL	scFv	$9.8 \times 10^{-3}$
VH1E4	2SD4 VL	scFv	$1.59 \times 10^{-2}$
VH1F6	2SD4 VL	scFv	$2.29 \times 10^{-2}$
VH1D8	2SD4 VL	scFv	$9.5 \times 10^{-3}$
VH1G1	2SD4 VL	scFv	$2.14 \times 10^{-2}$
2SD4 VH	EP B12	scFv	$6.7 \times 10^{-3}$
2SD4 VH	VL10E4	scFv	$9.6 \times 10^{-3}$
2SD4 VH	VL100A9	scFv	$1.33 \times 10^{-2}$
2SD4 VH	VL100D2	scFv	$1.41 \times 10^{-2}$
2SD4 VH	VL10F4	scFv	$1.11 \times 10^{-2}$
2SD4 VH	VLLOE5	scFv	$1.16 \times 10^{-2}$
2SD4 VH	VLL0F9	scFv	$6.09 \times 10^{-3}$
2SD4 VH	VLL0F10	scFv	$1.34 \times 10^{-2}$
2SD4 VH	VLLOG7	scFv	$1.56 \times 10^{-2}$
2SD4 VH	VLLOG9	scFv	$1.46 \times 10^{-2}$
2SD4 VH	VLLOH1	scFv	$1.17 \times 10^{-2}$
2SD4 VH	VLLOH10	scFv	$1.12 \times 10^{-2}$
2SD4 VH	VL1B7	scFv	$1.3 \times 10^{-2}$
2SD4 VH	VL1C1	scFv	$1.36 \times 10^{-2}$
2SD4 VH	VL1C7	scFv	$2.0 \times 10^{-2}$
2SD4 VH	VL0.1F4	scFv	$1.76 \times 10^{-2}$
2SD4 VH	VL0.1H8	scFv	$1.14 \times 10^{-2}$

- The slow off rates (*i.e.*,  $K_{\text{off}} \leq 1 \times 10^{-4} \text{ sec}^{-1}$ ) for full-length antibodies (*i.e.*, IgG format) having a VL selected from D2E7, LOE7, LOE7.T and LOE7.A, which have either a threonine or an alanine at position 9, indicate that position 9 of the D2E7 VL CDR3 can be occupied by either of these two residues without substantially affecting the  $K_{\text{off}}$ . Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Furthermore, the slow off rates (*i.e.*,  $K_{\text{off}} \leq 1 \times 10^{-4} \text{ sec}^{-1}$ ) for antibodies having a VH selected from D2E7, VH1-D2.N



and VH1-D2.Y, which have either a tyrosine or an asparagine at position 12, indicate that position 12 of the D2E7 VH CDR3 can be occupied by either of these two residues without substantially affecting the  $K_{\text{off}}$ . Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4).

The results shown in Table 6 demonstrate that, in scFv format, antibodies containing the 2SD4 VL or VH CDR3 region exhibit a faster  $K_{\text{off}}$  (i.e.,  $K_{\text{off}} \geq 1 \times 10^{-3} \text{ sec}^{-1}$ ) as compared to antibodies containing the D2E7 VL or VH CDR3 region. Within the VL CDR3, 2SD4 differs from D2E7 at positions 2, 5 and 9. As discussed above, however, position 9 may be occupied by Ala (as in 2SD4) or Thr (as in D2E7) without substantially affecting the  $K_{\text{off}}$ . Thus, by comparison of 2SD4 and D2E7, positions 2 and 5 of the D2E7 VL CDR3, both arginines, can be identified as being critical for the association of the antibody with hTNF $\alpha$ . These residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Regarding the importance of position 2, replacement of Arg (in LOE7, which has the same VL CDR3 as D2E7) with Lys (in EP B12) accelerates the off rate by a factor of two. Regarding the importance of position 5, replacement of Arg (in D2E7) with Ala (in LD2E7\*.A5), as described in Example 2, also accelerates the off rate two-fold. Furthermore, without either Arg at positions 2 and 5 (in 2SD4), the off rate is five-fold faster. However, it should be noted that although position 5 is important for improved binding to hTNF $\alpha$ , a change at this position can be negated by changes at other positions, as seen in VLLOE4, VLLOH1 or VL0.1H8.

Within the VH CDR3, 2SD4 differs from D2E7 at positions 1, 7 and 12. As discussed above, however, position 12 may be occupied by Asn (as in 2SD4) or Tyr (as in D2E7) without substantially affecting the  $K_{\text{off}}$ . Thus, by comparison of 2SD4 and D2E7, positions 1 and 7 of the D2E7 VH CDR3 can be identified as being critical for binding to hTNF $\alpha$ . As discussed above, these residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Both positions are important for binding to hTNF $\alpha$  since when the 3C-H2 VH CDR3 (which has a valine to alanine change at position 1 with respect to the D2E7 VH CDR3) is used, the scFv has a 3-fold faster off rate than when the D2E7 VH CDR3 is used but this off rate is still four times slower than when the 2SD4 VH CDR3 is used (which has changes at both positions 1 and 7 with respect to the D2E7 VH CDR3).

**EXAMPLE 4: Functional Activity of D2E7**

To examine the functional activity of D2E7, the antibody was used in several assays that measure the ability of the antibody to inhibit hTNF $\alpha$  activity, either *in vitro* or *in vivo*.

**A. Neutralization of TNF $\alpha$ -Induced Cytotoxicity in L929 Cells**

Human recombinant TNF $\alpha$  (rhTNF $\alpha$ ) causes cell cytotoxicity to murine L929 cells after an incubation period of 18-24 hours. Human anti-hTNF $\alpha$  antibodies were evaluated in L929 assays by coincubation of antibodies with rhTNF $\alpha$  and the cells as follows. A 96-well microtiter plate containing 100  $\mu$ l of anti-hTNF $\alpha$  Abs was serially diluted 1/3 down the plate in duplicates using RPMI medium containing 10% fetal bovine serum (FBS). Fifty microliters of rhTNF $\alpha$  was added for a final concentration of 500 pg/ml in each sample well. The plates were then incubated for 30 minutes at room temperature. Next, 50  $\mu$ l of TNF $\alpha$ -sensitive L929 mouse fibroblasts cells were added for a final concentration of  $5 \times 10^4$  cells per well, including 1  $\mu$ g/ml Actinomycin-D. Controls included medium plus cells and rhTNF $\alpha$  plus cells. These controls, and a TNF $\alpha$  standard curve, ranging from 2 ng/ml to 8.2 pg/ml, were used to determine the quality of the assay and provide a window of neutralization. The plates were then incubated overnight (18-24 hours) at 37° C in 5% CO<sub>2</sub>.

One hundred microliters of medium was removed from each well and 50  $\mu$ l of 5 mg/ml 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; commercially available from Sigma Chemical Co., St. Louis, MO) in PBS was added. The plates were then incubated for 4 hours at 37° C. Fifty microliters of 20% sodium dodecyl sulfate (SDS) was then added to each well and the plates were incubated overnight at 37° C. The optical density at 570/630 nm was measured, curves were plotted for each sample and IC<sub>50</sub>s were determined by standard methods.

Representative results for human antibodies having various VL and VH pairs, as compared to the murine MAK 195 mAb, are shown in Figure 3 and in Table 7 below.

**Table 7: Neutralization of TNF $\alpha$ -Induced L929 Cytotoxicity**

<u>VH</u>	<u>VL</u>	<u>Structure</u>	<u>IC<sub>50</sub>, M</u>
D2E7	D2E7	scFv	$1.1 \times 10^{-10}$
D2E7	D2E7	IgG4	$4.7 \times 10^{-11}$
2SD4	2SD4	scFv/IgG1/IgG4	$3.0 \times 10^{-7}$

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2SD4	LOE7	scFv	$4.3 \times 10^{-8}$
VH1-D2	2SD4	scFv	$1.0 \times 10^{-8}$
VH1-D2	LOE7	scFv/IgG1/IgG4	$3.4 \times 10^{-10}$
VH1.D2.Y	LOE7.T	IgG4	$8.1 \times 10^{-11}$
VH1-D2.N	LOE7.T	IgG4	$1.3 \times 10^{-10}$
VH1-D2.Y	LOE7.A	IgG4	$2.8 \times 10^{-11}$
VH1-D2.N	LOE7.A	IgG4	$6.2 \times 10^{-11}$
MAK 195	MAK 195	scFv	$1.9 \times 10^{-8}$
MAK 195	MAK195	F(ab') <sub>2</sub>	$6.2 \times 10^{-11}$

The results in Figure 3 and Table 7 demonstrate that the D2E7 human anti-hTNF $\alpha$  antibody, and various D2E7-related antibodies, neutralize TNF $\alpha$ -induced L929 cytotoxicity with a capacity approximately equivalent to that of the murine anti-hTNF $\alpha$  mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to neutralize TNF $\alpha$ -induced L929 cytotoxicity was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 8:

Table 8: Neutralization of TNF $\alpha$ -Induced L929 Cytotoxicity by D2E7 IgG1

<u>Experiment</u>	<u>IC<sub>50</sub> [M]</u>
1	$1.26 \times 10^{-10}$
2	$1.33 \times 10^{-10}$
3	$1.15 \times 10^{-10}$
Average	$1.25 \pm 0.01 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, neutralizes TNF $\alpha$ -induced L929 cytotoxicity with an average IC<sub>50</sub> [M] of  $1.25 \pm 0.01 \times 10^{-10}$ .

#### B. Inhibition of TNF $\alpha$ Binding to TNF $\alpha$ Receptors on U-937 Cells

The ability of human anti-hTNF $\alpha$  antibodies to inhibit the binding of hTNF $\alpha$  to hTNF $\alpha$  receptors on the surface of cells was examined using the U-937 cell line (ATCC No. CRL 1593), a human histiocytic cell line that expresses hTNF $\alpha$  receptors. U-937

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cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone A-1111, Hyclone Laboratories, Logan, UT), L-glutamine (4 mM), HEPES buffer solution (10 mM), penicillin (100 µg/ml) and streptomycin (100 µg/ml). To examine the activity of full-length IgG antibodies, U-937 cells were preincubated with PBS supplemented with 1 mg/ml of human IgG (Sigma I-4506, Sigma Chemical Co., St. Louis, MO) for 45 minutes on ice and then cells were washed three times with binding buffer. For the receptor binding assay, U-937 cells ( $5 \times 10^6$  cells/well) were incubated in a binding buffer (PBS supplemented with 0.2% bovine serum albumin) in 96-well microtiter plates (Costar 3799, Costar Corp., Cambridge, MA) together with  $^{125}\text{I}$ -labeled rhTNF $\alpha$  ( $3 \times 10^{-10}$  M; 25 µCi/ml; obtained from NEN Research Products, Wilmington, DE), with or without anti-hTNF $\alpha$  antibodies, in a total volume of 0.2 ml. The plates were incubated on ice for 1.5 hours. Then, 75 µl of each sample was transferred to 1.0 ml test tubes (Sarstedt 72.700, Sarstedt Corp., Princeton, NJ) containing dibutylphthalate (Sigma D-2270, Sigma Chemical Co., St. Louis, MO) and dinonylphthalate (ICN 210733, ICN, Irvine, CA). The test tubes contained a 300 µl mixture of dibutylphthalate and dinonylphthalate, 2:1 volume ratio, respectively. Free (*i.e.*, unbound)  $^{125}\text{I}$ -labeled rhTNF $\alpha$  was removed by microcentrifugation for five minutes. Then, each test tube end containing a cell pellet was cut with the aid of a microtube scissor (Bel-Art 210180001, Bel-Art Products, Pequannock, NJ). The cell pellet contains  $^{125}\text{I}$ -labeled rhTNF $\alpha$  bound to the p60 or p80 TNF $\alpha$  receptor, whereas the aqueous phase above the oil mixture contains excess free  $^{125}\text{I}$ -labeled rhTNF $\alpha$ . All cell pellets were collected in a counting tube (Falcon 2052, Becton Dickinson Labware, Lincoln Park, NJ) and counted in a scintillation counter.

Representative results are shown in Figure 4. The  $\text{IC}_{50}$  value for D2E7 inhibition of hTNF $\alpha$  binding to hTNF $\alpha$  receptors on U-937 cells is approximately  $3 \times 10^{-10}$  M in these experiments. These results demonstrate that the D2E7 human anti-hTNF $\alpha$  antibody inhibits rhTNF $\alpha$  binding to hTNF $\alpha$  receptors on U-937 cells at concentrations approximately equivalent to that of the murine anti-hTNF $\alpha$  mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit rhTNF $\alpha$  binding to hTNF $\alpha$  receptors on U-937 cells was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 9:

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Table 9: Inhibition of TNF Receptor Binding on U-937 Cells by D2E7 IgG1

Experiment	IC <sub>50</sub> [M]
1	1.70 x 10 <sup>-10</sup>
2	1.49 x 10 <sup>-10</sup>
3	1.50 x 10 <sup>-10</sup>
Average	1.56 ± 0.12 x 10 <sup>-10</sup>

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNF receptor binding on U-937 cells with an average IC<sub>50</sub> [M] of 1.56 ± 0.12 x 10<sup>-10</sup>.

To investigate the inhibitory potency of D2E7 in the binding of <sup>125</sup>I-rhTNF binding to individual p55 and p75 receptors, a solid phase radioimmunoassay was performed. To measure the IC<sub>50</sub> values of D2E7 for separate TNF receptors, varying concentrations of the antibody were incubated with 3 x 10<sup>-10</sup> concentration of <sup>125</sup>I-rhTNF. The mixture was then tested on separate plates containing either the p55 or the p75 TNF receptors in a dose dependent manner. The results are summarized below in Table 10:

Table 10: Inhibition of TNF Receptor Binding to p55 and p75 TNFR by D2E7 IgG1

Reagent	IC <sub>50</sub> [M]	
	p55 TNFR	p 75TNFR
D2E7	1.47 x 10 <sup>-9</sup>	1.26 x 10 <sup>-9</sup>
rhTNF	2.31 x 10 <sup>-9</sup>	2.70 x 10 <sup>-9</sup>

Inhibition of <sup>125</sup>I-rhTNF binding to the p55 and p75 TNF receptors on U937 cells by D2E7 followed a simple sigmoidal curve, indicating similar IC<sub>50</sub> values for each receptor. In the solid phase radioimmunoassay (RIA) experiments with recombinant TNF receptors, IC<sub>50</sub> values for inhibition of <sup>125</sup>I-rhTNF binding to the p55 and the p75 receptors by D2E7 were calculated as 1.47 x 10<sup>-9</sup> and 1.26 x 10<sup>-9</sup> M, respectively. The decrease in IC<sub>50</sub> values in the solid phase was probably due to higher density of receptors in the RIA format, as unlabeled rhTNF also inhibited with similar IC<sub>50</sub> values. The IC<sub>50</sub> values for inhibition of <sup>125</sup>I-rhTNF binding to the p55 and the p75 receptors by unlabeled rhTNF were 2.31 x 10<sup>-9</sup> and 2.70 x 10<sup>-9</sup> M, respectively

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C. Inhibition of ELAM-1 Expression on HUVEC

Human umbilical vein endothelial cells (HUVEC) can be induced to express endothelial cell leukocyte adhesion molecule 1 (ELAM-1) on their cell-surface by treatment with rhTNF $\alpha$ , which can be detected by reacting rhTNF $\alpha$ -treated HUVEC with an mouse anti-human ELAM-1 antibody. The ability of human anti-hTNF $\alpha$  antibodies to inhibit this TNF $\alpha$ -induced expression of ELAM-1 on HUVEC was examined as follows: HUVEC (ATCC No. CRL 1730) were plated in 96-well plates ( $5 \times 10^4$  cells/well) and incubated overnight at 37 °C. The following day, serial dilutions of human anti-hTNF $\alpha$  antibody (1:10) were prepared in a microtiter plate, starting with 20-100  $\mu$ g/ml of antibody. A stock solution of rhTNF $\alpha$  was prepared at 4.5 ng/ml, aliquots of rhTNF $\alpha$  were added to each antibody-containing well and the contents were mixed well. Controls included medium alone, medium plus anti-hTNF $\alpha$  antibody and medium plus rhTNF $\alpha$ . The HUVEC plates were removed from their overnight incubation at 37° C and the medium gently aspirated from each well. Two hundred microliters of the antibody-rhTNF $\alpha$  mixture were transferred to each well of the HUVEC plates. The HUVEC plates were then further incubated at 37° C for 4 hours. Next, a murine anti-ELAM-1 antibody stock was diluted 1:1000 in RPMI. The medium in each well of the HUVEC plate was gently aspirated, 50  $\mu$ l/well of the anti-ELAM-1 antibody solution was added and the HUVEC plates were incubated 60 minutes at room temperature. An  $^{125}$ I-labeled anti-mouse Ig antibody solution was prepared in RPMI (approximately 50,000 cpm in 50  $\mu$ l). The medium in each well of the HUVEC plates was gently aspirated, the wells were washed twice with RPMI and 50  $\mu$ l of the  $^{125}$ I-labeled anti-mouse Ig solution was added to each well. The plates were incubated for one hour at room temperature and then each well was washed three times with RPMI. One hundred eighty microliters of 5% SDS was added to each well to lyse the cells. The cell lysate from each well was then transferred to a tube and counted in a scintillation counter.

Representative results are shown in Figure 5. The IC<sub>50</sub> value for D2E7 inhibition of hTNF $\alpha$ -induced expression of ELAM-1 on HUVEC is approximately  $6 \times 10^{-11}$  M in these experiments. These results demonstrate that the D2E7 human anti-hTNF $\alpha$  antibody inhibits the hTNF $\alpha$ -induced expression of ELAM-1 on HUVEC at concentrations approximately equivalent to that of the murine anti-hTNF $\alpha$  mAb MAK 195.

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In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit hTNF $\alpha$ -induced expression of ELAM-1 on HUVEC was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 11:

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Table 11: Inhibition of TNF $\alpha$ -Induced ELAM-1 Expression by D2E7 IgG1 Receptor

Experiment	IC <sub>50</sub> [M]
1	1.95 x 10 <sup>-10</sup>
2	1.69 x 10 <sup>-10</sup>
3	1.90 x 10 <sup>-10</sup>
Average	1.85 $\pm$ 0.14 x 10 <sup>-10</sup>

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNF $\alpha$ -induced ELAM-1 expression on HUVEC with an average IC<sub>50</sub> [M] of  
10 1.85  $\pm$  0.14 x 10<sup>-10</sup>.

The neutralization potency of D2E7 IgG1 was also examined for the rhTNF induced expression of two other adhesion molecules, ICAM-1 and VCAM-1. Since the rhTNF titration curve for ICAM-1 expression at 16 hours was very similar to the curve  
15 of ELAM-1 expression, the same concentration of rhTNF was used in the antibody neutralization experiments. The HUVEC were incubated with rhTNF in the presence of varying concentrations of D2E7 in a 37°C CO<sub>2</sub> incubator for 16 hours, and the ICAM-1 expression was measured by mouse anti-ICAM-1 antibody followed by <sup>125</sup>I-labeled sheep anti-mouse antibody. Two independent experiments were performed and the IC<sub>50</sub>  
20 values were calculated. An unrelated human IgG1 antibody did not inhibit the ICAM-1 expression.

The experimental procedure to test inhibition of VCAM-1 expression was the same as the procedure for ELAM-1 expression, except anti-VCAM-1 MAb was used instead of anti-ELAM-1 MAb. Three independent experiments were performed and the  
25 IC<sub>50</sub> values were calculated. An unrelated human IgG1 antibody did not inhibit VCAM-1 expression.

The results are summarized below in Table 12:

Table 12: Inhibition of ICAM-1 and VCAM-1 Expression by D2E7 IgG1

ICAM-1 Inhibition		IC <sub>50</sub> [M]	
Experiment	IC <sub>50</sub> [M]	Experiment	IC <sub>50</sub> [M]
1	$1.84 \times 10^{-10}$	1	$1.03 \times 10^{-10}$
2	$2.49 \times 10^{-10}$	2	$9.26 \times 10^{-11}$
		3	$1.06 \times 10^{-10}$
Average	$2.17 \pm 0.46 \times 10^{-10}$	Average	$1.01 \pm 0.01 \times 10^{-10}$

These experiments demonstrate that treatment of primary human umbilical vein endothelial cells with rhTNF led to optimum expression of adhesion molecules:

- 5 ELAM-1 and VCAM-1 at four hours, and the maximum up-regulated expression of ICAM-1 at 16 hours. D2E7 was able to inhibit the expression of the three adhesion molecules in a dose dependent manner. The IC<sub>50</sub> values for the inhibition of ELAM-1, ICAM-1 and VCAM-1 were  $1.85 \times 10^{-10}$ ,  $2.17 \times 10^{-10}$  and  $1.01 \times 10^{-10}$  M, respectively. These values are very similar, indicating similar requirements for the dose of rhTNF
- 10 activation signal to induce ELAM-1, ICAM-1 and VCAM-1 expression. Interestingly, D2E7 was similarly effective in the longer inhibition assay of the the ICAM-1 expression. The ICAM-1 inhibition assay required 16 hours of co-incubation of rhTNF and D2E7 with HUVEC as opposed to 4 hours required for the ELAM-1 and the VCAM-1 inhibition assays. Since D2E7 has a slow off-rate for rhTNF, it is conceivable
- 15 that during the 16 hour co-incubation period there was no significant competition by the TNF receptors on the HUVEC.

#### D. In Vivo Neutralization of hTNF $\alpha$

- 20 Three different *in vivo* systems were used to demonstrate that D2E7 is effective at inhibiting hTNF $\alpha$  activity *in vivo*.

##### I. Inhibition of TNF-Induced Lethality in D-Galactosamine-Sensitized Mice

- 25 Injection of recombinant human TNF $\alpha$  (rhTNF $\alpha$ ) to D-galactosamine sensitized mice causes lethality within a 24 hour time period. TNF $\alpha$  neutralizing agents have been shown to prevent lethality in this model. To examine the ability of human anti-hTNF $\alpha$  antibodies to neutralize hTNF $\alpha$  *in vivo* in this model, C57Bl/6 mice were injected with varying concentrations of D2E7-IgG1, or a control protein, in PBS intraperitoneally
- 30 (i.p.). Mice were challenged 30 minutes later with 1  $\mu$ g of rhTNF $\alpha$  and 20 mg of D-



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galactosamine in PBS i.p., and observed 24 hours later. These amount of rhTNF $\alpha$  and D-galactosamine were previously determined to achieve 80-90% lethality in these mice.

Representative results, depicted as a bar graph of % survival versus antibody concentration, are shown in Figure 6. The black bars represent D2E7, whereas the hatched bars represent MAK 195. Injection of 2.5-25  $\mu$ g of D2E7 antibody per mouse protected the animals from TNF $\alpha$ -induced lethality. The ED<sub>50</sub> value is approximately 1-2.5  $\mu$ g/mouse. The positive control antibody, MAK 195, was similar in its protective ability. Injection of D2E7 in the absence of rhTNF $\alpha$  did not have any detrimental effect on the mice. Injection of a non-specific human IgG1 antibody did not offer any protection from TNF $\alpha$ -induced lethality.

In a second experiment, forty-nine mice were divided into 7 equal groups. Each group received varying doses of D2E7 thirty minutes prior to receiving an LD<sub>80</sub> dose of rhTNF/D-galactosamine mixture (1.0  $\mu$ g rhTNF and 20 mg D-galactosamine per mouse). Control group 7 received normal human IgG1 kappa antibody at 25  $\mu$ g/mouse dose. The mice were examined 24 hours later. Survival for each group is summarized below in Table 13.

Table 13: 24 Hour Survival After Treatment with D2E7

Group	Survival (alive/total)	Survival (%)
1 (no antibody)	0/7	0
2 (1 $\mu$ g)	1/7	14
3 (2.6 $\mu$ g)	5/7	71
4 (5.2 $\mu$ g)	6/7	86
5 (26 $\mu$ g)	6/7	86
6 (26 $\mu$ g; no rhTNF)	7/7	100
7 (25 $\mu$ g Hu IgG1)	1/7	14

## II. Inhibition of TNF-Induced Rabbit Pyrexia

The efficacy of D2E7 in inhibiting rhTNF-induced pyrexia response in rabbits was examined. Groups of three NZW female rabbits weighing approximately 2.5 kg each were injected intravenously with D2E7, rhTNF, and immune complexes of D2E7 and rhTNF. Rectal temperatures were measured by thermistor probes on a Kaye thermal recorder every minute for approximately 4 hours. Recombinant human TNF in saline, injected at 5  $\mu$ g/kg, elicited a rise in temperature greater than 0.4°C at approximately 45

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minutes after injection. The antibody preparation by itself, in saline at a dose of 138 µg/kg, did not elicit a rise in temperature in the rabbits up to 140 minutes after administration. In all further experiments, D2E7 or control reagents (human IgG1 or a saline vehicle) were injected i.v. into rabbits followed 15 minutes later by an injection of rhTNF in saline at 5 µg/kg i.v. Representative results of several experiments are summarized below in Table 14:

**Table 14: Inhibition of rhTNF-induced Pyrexia with D2E7 in Rabbits**

D2E7 dose (µg/kg)	Temp. rise*, °C		% Inhib.**	Molar Ratio D2E7: rhTNF	Peak Temp. minutes post rhTNF
	rhTNF	rhTNF + D2E7			
14	0.53	0.25	53	1	60
24	0.43	0.13	70	1.6	40
48	0.53	0.03	94	3.3	50
137	0.53	0.00	100	9.5	60
792	0.80	0.00	100	55	60

\*=Peak temperature

10 \*\*=% inhibition=(1-{temperature rise with rhTNF & D2E7/temperature rise with rhTNF alone}) x 100.

Intravenous pretreatment with D2E7 at a dose of 14 µg/kg partially inhibited the pyrogenic response, compared to rabbits pre-treated with saline alone. D2E7 administered at 137 µg/kg totally suppressed the pyrogenic response of rhTNF in the same experiment. In a second experiment, D2E7 administered at 24 µg/kg also partially suppressed the pyrogenic response, compared to rabbits pretreated with saline alone. The molar ratio of D2E7 to rhTNF was 1/6:1 in this experiment. In a third experiment, D2E7 injected i.v. at 48 µg/kg (molar ratio D2E7:rhTNF = 3.3:1) totally suppressed the pyrogenic response, compared to rabbits pretreated with the control human IgG1 in saline at 30 µg/kg. In the final experiment, rabbits pretreated with D2E7 (792 µg/kg) at a very high molar ratio to rhTNF (55:1) did not develop any rise in temperature at any time up to 4 hours of observation. Treatment of rabbits with immune complexes generated from a mixture of D2E7 and rhTNF incubated at 37°C for 1 hour at a molar ratio of 55:1, without subsequent rhTNF administration, also did not elicit any rise in temperature in the same experiment.

### III. Prevention of Polyarthrititis in Tg197 Transgenic Mice

The effect of D2E7 on disease development was investigated in a transgenic murine model of arthritis. Transgenic mice (Tg197) have been generated that express human wild type TNF (modified in the 3' region beyond the coding sequences) and these mice develop chronic polyarthrititis with 100% incidence at 4-7 weeks of age (see *EMBO J* (1991) 10:4025-4031 for further description of the Tg197 model of polyarthrititis).

Transgenic animals were identified by PCR at 3 days of age. Litters of transgenic mice were divided into six groups. Transgenic mice were verified by slot-blot hybridization analysis at 15 days of age. The treatment protocols for the six groups were as follows: Group 1=no treatment; Group 2=saline (vehicle); Group 3=D2E7 at 1.5 µg/g; Group 4=D2E7 at 15 µg/g; Group 5=D2E7 at 30 µg/g; and Group 6=IgG1 isotype control at 30 µg/g. A litter with non transgenic mice was also included in the study to serve as a control (Group 7 - nontransgenic; no treatment). Each group received three i.p. injections per week of the indicated treatments. Injections continued for 10 weeks. Each week, macroscopic changes in joint morphology were recorded for each animal. At 10 weeks, all mice were sacrificed and mouse tissue was collected in formalin. Microscopic examination of the tissue was performed.

Animal weight in grams was taken for each mouse at the start of each week. At the same time measurements of joint size (in mm) were also taken, as a measurement of disease severity. Joint size was established as an average of three measurements on the hind right ankle using a micrometer device. Arthritic scores were recorded weekly as follows: 0 = No arthritis, (normal appearance and flexion); + = mild arthritis (joint distortion); ++ = moderate arthritis (swelling, joint deformation) and +++ = heavy arthritis (ankylosis detected on flexion and severely impaired movement).

Histopathological scoring based on haematoxylin/eosin staining of joint sections was based as follows; 0 = No detectable disease; 1 = proliferation of the synovial membrane; 2 = heavy synovial thickening 3 = cartilage destruction and bone erosion.

The effect of D2E7 treatment on the mean joint size of the Tg197 transgenic arthritic mice is shown in the graph of Figure 9. The histopathological and arthritic scores of the Tg197 transgenic mice, at 11 weeks of age, are summarized below in Table 15:

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Table 15: Effect of D2E7 on Histopathology and Arthritic Score in Tg197 Mice

Group	Treatment	Histopathological Score	Arthritic Score
1	none	3 (7/70)	+++ (7/7)
2	saline	3 (8/8)	+++ (8/8)
6	IgG1 control	3 (9/9)	+++ (7/9)
3	D2E7 at 1.5 µg/g	0 (6/8)	0 (8/8)
4	D2E7 at 15 µg/g	0 (7/8)	0 (8/8)
5	D2E7 at 30 µg/g	0 (8/8)	0 (8/8)

This experiment demonstrated that the D2E7 antibody has a definite beneficial effect on transgenic mice expressing the wild-type human TNF (Tg197) with no arthritis evident after the study period.

#### E. D2E7 Neutralization of TNF $\alpha$ s from Other Species

The binding specificity of D2E7 was examined by measuring its ability to neutralize tumor necrosis factors from various primate species and from mouse, using an L929 cytotoxicity assay (as described in Example 4, subsection A, above). The results are summarized in Table 16 below:

Table 16: Ability of D2E7 to Neutralize TNF from Different Species in the L929 Assay

<u>TNF<math>\alpha</math>*</u>	<u>Source</u>	<u>IC<sub>50</sub> for D2E7 Neutralization (M)**</u>
Human	Recombinant	$7.8 \times 10^{-11}$
Chimpanzee	LPS-stimulated PBMC	$5.5 \times 10^{-11}$
baboon	Recombinant	$6.0 \times 10^{-11}$
marmoset	LPS-stimulated PBMC	$4.0 \times 10^{-10}$
cynomolgus	LPS-stimulated PBMC	$8.0 \times 10^{-11}$
rhesus	LPS-stimulated PBMC	$3.0 \times 10^{-11}$
canine	LPS-stimulated WBC	$2.2 \times 10^{-10}$
porcine	Recombinant	$1.0 \times 10^{-7}$
murine	Recombinant	$>1.0 \times 10^{-7}$

The results in Table 16 demonstrate that D2E7 can neutralize the activity of five primate TNF $\alpha$ s approximately equivalently to human TNF $\alpha$  and, moreover, can

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neutralize the activity of canine TNF $\alpha$  (about ten-fold less well than human TNF $\alpha$ ) and porcine and mouse TNF $\alpha$  (about ~1000-fold less well than human TNF $\alpha$ ). Moreover, the binding of D2E7 to solution phase rhTNF $\alpha$  was not inhibited by other cytokines, such as lymphotoxin (TNF $\beta$ ), IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IFN $\gamma$  and TGF $\beta$ ,  
5 indicating that D2E7 is very specific for its ligand TNF $\alpha$ .

F. Lack of Cytokine Release by Human Whole Blood Incubated with D2E7

In this example, the ability of D2E7 to induce, by itself, normal human blood  
10 cells to secrete cytokines or shed cell surface molecules was examined. D2E7 was incubated with diluted whole blood from three different normal donors at varying concentrations for 24 hours. An LPS positive control was run at the same time, at a concentration previously determined to stimulate immunocompetent blood cells to secrete cytokines. The supernatants were harvested and tested in a panel of ten soluble  
15 cytokine, receptor and adhesion molecule ELISA kits: IL-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor antagonist, IL-6, IL-8, TNF $\alpha$ , soluble TNF receptor I, soluble TNF receptor II, soluble ICAM-1 and soluble E-selectin. No significant amounts of cytokines or shed cell surface molecules were measured as a result of D2E7 antibody co-incubation, at concentrations up to 343  $\mu$ g/ml. Control cultures without the addition of the antibody  
20 also did not yield any measurable amounts of cytokines, whereas the LPS co-culture control yielded elevated values in the high picogram to low nanogram range. These results indicate that D2E7 did not induce whole blood cells to secrete cytokines or shed cell surface proteins above normal levels in *ex vivo* cultures.

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Forming part of the present disclosure is the appended Sequence Listing, the contents of which are summarized in the table below:

SEQ ID NO:	ANTIBODY CHAIN	REGION	SEQUENCE TYPE
1	D2E7	VL	amino acid
2	D2E7	VH	amino acid
3	D2E7	VL CDR3	amino acid
4	D2E7	VH CDR3	amino acid
5	D2E7	VL CDR2	amino acid
6	D2E7	VH CDR2	amino acid
7	D2E7	VL CDR1	amino acid
8	D2E7	VH CDR1	amino acid
9	2SD4	VL	amino acid
10	2SD4	VH	amino acid
11	2SD4	VL CDR3	amino acid
12	EP B12	VL CDR3	amino acid
13	VL10E4	VL CDR3	amino acid
14	VL100A9	VL CDR3	amino acid
15	VLL100D2	VL CDR3	amino acid
16	VLL0F4	VL CDR3	amino acid
17	LOE5	VL CDR3	amino acid
18	VLLOG7	VL CDR3	amino acid
19	VLLOG9	VL CDR3	amino acid
20	VLL0H1	VL CDR3	amino acid
21	VLL0H10	VL CDR3	amino acid
22	VL1B7	VL CDR3	amino acid
23	VL1C1	VL CDR3	amino acid
24	VL0.1F4	VL CDR3	amino acid
25	VL0.1H8	VL CDR3	amino acid
26	LOE7.A	VL CDR3	amino acid
27	2SD4	VH CDR3	amino acid
28	VH1B11	VH CDR3	amino acid
29	VH1D8	VH CDR3	amino acid
30	VH1A11	VH CDR3	amino acid
31	VH1B12	VH CDR3	amino acid
32	VH1E4	VH CDR3	amino acid
33	VH1F6	VH CDR3	amino acid
34	3C-H2	VH CDR3	amino acid
35	VH1-D2.N	VH CDR3	amino acid
36	D2E7	VL	nucleic acid
37	D2E7	VH	nucleic acid

EQUIVALENTS

- Those skilled in the art will recognize, or be able to ascertain using no more than
- 5 routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT:  
(A) NAME: BASF Aktiengesellschaft  
(B) STREET: Carl-Bosch Str. 38  
(C) CITY: 67056 Ludwigshafen  
(D) STATE: Rheinland-Pfalz  
10 (E) COUNTRY: Federal Republic of Germany
- (ii) TITLE OF INVENTION: Human Antibodies that Bind Human TNF $\alpha$
- (iii) NUMBER OF SEQUENCES: 37  
15
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: LAHIVE & COCKFIELD  
(B) STREET: 60 State Street, suite 510  
(C) CITY: Boston  
20 (D) STATE: Massachusetts  
(E) COUNTRY: USA  
(F) ZIP: 02109-1875
- (v) COMPUTER READABLE FORM:  
25 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 30 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- 35 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 08/599,226  
(B) FILING DATE: 09-FEB-1996  
(C) CLASSIFICATION:
- 40 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 60/031,476  
(B) FILING DATE: 25-NOV-1996  
(C) CLASSIFICATION:
- 45 (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: DeConti, Giulio A., Jr.  
(B) REGISTRATION NUMBER: 31,503  
(C) REFERENCE/DOCKET NUMBER: BBI-043CPPC
- 50 (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (617)227-7400  
(B) TELEFAX: (617)227-5941

55 (2) INFORMATION FOR SEQ ID NO:1:



- 63 -

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 107 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5				10						15	
Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Gly	Ile	Arg	Asn	Tyr
			20					25					30		
Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys	Leu	Leu	Ile
			35				40					45			
Tyr	Ala	Ala	Ser	Thr	Leu	Gln	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
			50				55				60				
Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro
			65			70				75				80	
Glu	Asp	Val	Ala	Thr	Tyr	Tyr	Cys	Gln	Arg	Tyr	Asn	Arg	Ala	Pro	Tyr
				85				90						95	
Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu	Ile	Lys					
			100					105							

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 121 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Arg
1				5				10						15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Asp	Asp	Tyr
			20					25					30		
Ala	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val
			35				40					45			

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Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val  
 50 55 60  
 5 Glu Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 10 Ala Lys Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Tyr Trp Gly  
 100 105 110  
 Gln Gly Thr Leu Val Thr Val Ser Ser  
 15 115 120

## (2) INFORMATION FOR SEQ ID NO:3:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 25 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal  
 (ix) FEATURE:  
 30 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 9  
 (D) OTHER INFORMATION: /note= "Xaa is Thr or Ala"  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
 35 Gln Arg Tyr Asn Arg Ala Pro Tyr Xaa  
 1 5

## 40 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12 amino acids  
 (B) TYPE: amino acid  
 45 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal  
 50 (ix) FEATURE:  
 (A) NAME/KEY: Modified-site  
 (B) LOCATION: 12  
 (D) OTHER INFORMATION: /note= "Xaa is Tyr or Asn"  
 55

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Xaa  
 1 5 10

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 7 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20 Ala Ala Ser Thr Leu Gln Ser  
 1 5

(2) INFORMATION FOR SEQ ID NO:6:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val Glu  
 1 5 10 15

40

Gly

(2) INFORMATION FOR SEQ ID NO:7:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Arg Ala Ser Gln Gly Ile Arg Asn Tyr Leu Ala  
1 5 10

5

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

15

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20

Asp Tyr Ala Met His  
1 5

## (2) INFORMATION FOR SEQ ID NO:9:

25

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

30

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Ile Gly  
1 5 10 15

40

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Tyr  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
35 40 45

45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

50

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Lys Tyr Asn Ser Ala Pro Tyr  
85 90 95

55

Ala Phe Gly Gln Gly Thr Lys Val Glu Ile Lys  
100 105

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## (2) INFORMATION FOR SEQ ID NO:10:

- 5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 121 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

15 Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg  
 1 5 10 15  
 20 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr  
 20 20 25 30  
 Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Asp Trp Val  
 35 40 45  
 25 Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val  
 50 55 60  
 Glu Gly Arg Phe Ala Val Ser Arg Asp Asn Ala Lys Asn Ala Leu Tyr  
 65 70 75 80  
 30 Leu Gln Met Asn Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95  
 Thr Lys Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn Trp Gly  
 35 100 105 110  
 Gln Gly Thr Leu Val Thr Val Ser Ser  
 115 120

40

## (2) INFORMATION FOR SEQ ID NO:11:

- 45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

55 Gln Lys Tyr Asn Ser Ala Pro Tyr Ala  
 1 5

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## (2) INFORMATION FOR SEQ ID NO:12:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gln Lys Tyr Asn Arg Ala Pro Tyr Ala  
1 5

20 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gln Lys Tyr Gln Arg Ala Pro Tyr Thr  
1 5

35

(2) INFORMATION FOR SEQ ID NO:14:

40 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

50 Gln Lys Tyr Ser Ser Ala Pro Tyr Thr  
1 5

55 (2) INFORMATION FOR SEQ ID NO:15:

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- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
- Gln Lys Tyr Asn Ser Ala Pro Tyr Thr  
1 5
- 15 (2) INFORMATION FOR SEQ ID NO:16:
- (i) SEQUENCE CHARACTERISTICS:  
20 (A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 25 (v) FRAGMENT TYPE: internal
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
- Gln Lys Tyr Asn Arg Ala Pro Tyr Thr  
30 1 5
- (2) INFORMATION FOR SEQ ID NO:17:
- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
- 45 Gln Lys Tyr Asn Ser Ala Pro Tyr Tyr  
1 5
- 50 (2) INFORMATION FOR SEQ ID NO:18:
- (i) SEQUENCE CHARACTERISTICS:  
55 (A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide  
(v) FRAGMENT TYPE: internal  
5  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:  
Gln Lys Tyr Asn Ser Ala Pro Tyr Asn  
1 5

10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide  
20

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25 Gln Lys Tyr Thr Ser Ala Pro Tyr Thr  
1 5

(2) INFORMATION FOR SEQ ID NO:20:

30

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gln Lys Tyr Asn Arg Ala Pro Tyr Asn  
1 5

45

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
50 (B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

55 (v) FRAGMENT TYPE: internal



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

5       Gln Lys Tyr Asn Ser Ala Ala Tyr Ser  
      1                               5

(2) INFORMATION FOR SEQ ID NO:22:

10       (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 9 amino acids  
          (B) TYPE: amino acid  
          (D) TOPOLOGY: linear

15       (ii) MOLECULE TYPE: peptide

          (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

20       Gln Gln Tyr Asn Ser Ala Pro Asp Thr  
      1                               5

(2) INFORMATION FOR SEQ ID NO:23:

30       (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 9 amino acids  
          (B) TYPE: amino acid  
          (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: peptide

35       (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

40       Gln Lys Tyr Asn Ser Asp Pro Tyr Thr  
      1                               5

(2) INFORMATION FOR SEQ ID NO:24:

45       (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 9 amino acids  
          (B) TYPE: amino acid  
          (D) TOPOLOGY: linear

50       (ii) MOLECULE TYPE: peptide

          (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

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Gln Lys Tyr Ile Ser Ala Pro Tyr Thr  
1 5

## 5 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal  
15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gln Lys Tyr Asn Arg Pro Pro Tyr Thr  
1 5  
20

## (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:  
25 (A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide  
30

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gln Arg Tyr Asn Arg Ala Pro Tyr Ala  
1 5  
35

## 40 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn  
1 5 10  
55

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## (2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12 amino acids  
 5 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
  
 (ii) MOLECULE TYPE: peptide  
  
 10 (v) FRAGMENT TYPE: internal  
  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:  
  
 Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Lys  
 15 1 5 10

## (2) INFORMATION FOR SEQ ID NO:29:

20 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
  
 25 (ii) MOLECULE TYPE: peptide  
  
 (v) FRAGMENT TYPE: internal  
  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:  
 30 Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Tyr  
 1 5 10

## 35 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12 amino acids  
 (B) TYPE: amino acid  
 40 (D) TOPOLOGY: linear  
  
 (ii) MOLECULE TYPE: peptide  
  
 (v) FRAGMENT TYPE: internal  
 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:  
  
 Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asp  
 50 1 5 10

## (2) INFORMATION FOR SEQ ID NO:31:

55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 12 amino acids

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(B) TYPE: amino acid  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide  
(v) FRAGMENT TYPE: internal  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
10 Ala Ser Tyr Leu Ser Thr Ser Phe Ser Leu Asp Tyr  
1 5 10

15 (2) INFORMATION FOR SEQ ID NO:32:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide  
(v) FRAGMENT TYPE: internal  
25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:  
Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu His Tyr  
1 5 10

30 (2) INFORMATION FOR SEQ ID NO:33:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
35 (B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide  
40 (v) FRAGMENT TYPE: internal  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:  
Ala Ser Phe Leu Ser Thr Ser Ser Ser Leu Glu Tyr  
45 1 5 10

(2) INFORMATION FOR SEQ ID NO:34:  
50 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: peptide

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(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

5

Ala Ser Tyr Leu Ser Thr Ala Ser Ser Leu Glu Tyr  
 1 5 10

10 (2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Asn  
 1 5 10

25

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 321 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

40 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGGGA CAGAGTCACC 60  
 ATCACTTGTC GGGCAAGTCA GGGCATCAGA AATTACTTAG CCTGGTATCA GCAAAAACCA 120  
 GGGAAAGCCC CTAAGCTCCT GATCTATGCT GCATCCACTT TGCAATCAGG GGTCCCATCT 180  
 45 CGGTTCAAGTGC GCAGTGGATC TGGGACAGAT TTCACTCTCA CCATCAGCAG CCTACAGCCT 240  
 GAAGATGTTG CAACTTATTA CTGTCAAAGG TATAACCGTG CACCGTATAC TTTTGGCCAG 300  
 GGGACCAAGG TGGAAATCAA A 321  
 50

(2) INFORMATION FOR SEQ ID NO 37:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 363 base pairs

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(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

	GAGGTGCAGC TGGTGGAGTC TGGGGGAGGC TTGGTACAGC CCGGCAGGTC CCTGAGACTC	60
10	TCCTGTGCGG CCTCTGGATT CACCTTTGAT GATTATGCCA TGCACTGGGT CCGGCAAGCT	120
	CCAGGGAAGG GCCTGGAATG GGTCTCAGCT ATCACTTGA ATAGTGGTCA CATAGACTAT	180
15	GCGGACTCTG TGGAGGGCCG ATTCACCATC TCCAGAGACA ACGCCAAGAA CTCCTGTAT	240
	CTGCAAATGA ACAGTCTGAG AGCTGAGGAT ACGGCCGTAT ATTACTGTGC GAAAGTCTCG	300
	TACCTTAGCA CCGCGTCCTC CTTGACTAT TGGGGCCAAG GTACCCTGGT CACCGTCTCG	360
20	AGT	363

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CLAIMS

1. An isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNF $\alpha$  with a  $K_d$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant  
5 of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, both determined by surface plasmon resonance, and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-7}$  M or less.
2. The isolated human antibody, or antigen-binding portion thereof, of claim  
10 1, which dissociates from human TNF $\alpha$  with a  $K_{off}$  rate constant of  $5 \times 10^{-4} \text{ s}^{-1}$  or less, .
3. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which dissociates from human TNF $\alpha$  with a  $K_{off}$  rate constant of  $1 \times 10^{-4} \text{ s}^{-1}$  or less.
- 15 4. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  
 $IC_{50}$  of  $1 \times 10^{-8}$  M or less.
- 20 5. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  
 $IC_{50}$  of  $1 \times 10^{-9}$  M or less.
6. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  
25  $IC_{50}$  of  $5 \times 10^{-10}$  M or less.
7. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which is a recombinant antibody, or antigen-binding portion thereof.
- 30 8. The isolated human antibody, or antigen-binding portion thereof, of claim  
1, which inhibits human TNF $\alpha$ -induced expression of ELAM-1 on human umbilical  
vein endothelial cells.
9. An isolated human antibody, or antigen-binding portion thereof, with the  
35 following characteristics:

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- a) dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance;
- b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;
- c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.
10. The isolated human antibody of claim 9, or an antigen-binding portion thereof, which dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  rate constant of  $5 \times 10^{-4} \text{ s}^{-1}$  or less.
11. The isolated human antibody of claim 9, or an antigen-binding portion thereof, which dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  rate constant of  $1 \times 10^{-4} \text{ s}^{-1}$  or less.
12. An isolated human antibody, or an antigen-binding portion thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11.
13. The isolated human antibody, or an antigen-binding portion thereof, of claim 12, wherein the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6.
14. The isolated human antibody, or an antigen-binding portion thereof, of claim 13, wherein the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8.



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15. An isolated human antibody, or an antigen binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of  
5 SEQ ID NO: 2.

16. The isolated human antibody of claim 15, which has an IgG1 heavy chain constant region.

10 17. The isolated human antibody of claim 15, which has an IgG4 heavy chain constant region.

18. The isolated human antibody of claim 15, which is a Fab fragment.

15 19. The isolated human antibody of claim 15, which is a single chain Fv fragment.

20. An isolated human antibody, or an antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid  
20 sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or with a  
25 heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34.

21. A recombinant human antibody, or antigen-binding portion thereof, that  
30 neutralizes the activity of human TNF $\alpha$  but not human TNF $\beta$ .

22. The recombinant human antibody, or antigen-binding portion thereof, of claim 21, which also neutralizes the activity of chimpanzee TNF $\alpha$  and at least one additional primate TNF $\alpha$  selected from the group consisting of baboon TNF $\alpha$ , marmoset  
35 TNF $\alpha$ , cynomolgus TNF $\alpha$  and rhesus TNF $\alpha$ .

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23. The recombinant human antibody, or an antigen-binding portion thereof, of claim 22, which also neutralizes the activity of canine TNF $\alpha$ .

24. The recombinant human antibody, or an antigen-binding portion thereof,  
5 of claim 22, which also neutralizes the activity of pig TNF $\alpha$ .

25. An isolated nucleic acid encoding a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, or by one to five conservative amino acid  
10 substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9.

26. The isolated nucleic acid of claim 25, which encodes an antibody light chain variable region (LCVR).

15 27. The isolated nucleic acid of claim 26, wherein the CDR2 domain of the antibody LCVR comprises the amino acid sequence of SEQ ID NO: 5.

28. The isolated nucleic acid of claim 27, wherein the CDR1 domain of the antibody LCVR comprises the amino acid sequence of SEQ ID NO: 7.  
20

29. An isolated nucleic acid encoding a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11, or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.  
25

30. The isolated nucleic acid of claim 29, which encodes an antibody heavy chain variable region (HCVR).

31. The isolated nucleic acid of claim 30, wherein the CDR2 domain of the  
30 antibody HCVR comprises the amino acid sequence of SEQ ID NO: 6.

32. The isolated nucleic acid of claim 31, wherein the CDR1 domain of the antibody HCVR comprises the amino acid sequence of SEQ ID NO: 8.

33. An isolated nucleic acid encoding a CDR3 domain comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO 4,  
35

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SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15,  
SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20,  
SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25,  
SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30,  
5 SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and SEQ ID NO: 34.

34. An isolated nucleic acid encoding an antibody light chain variable region comprising the amino acid sequence of SEQ ID NO: 1.

10 35. The isolated nucleic acid of claim 34, which encodes the antibody light chain variable region and an antibody light chain constant region.

36. The isolated nucleic acid of claim 35, which is in a recombinant expression vector.

15

37. An isolated nucleic acid encoding an antibody heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2.

38. The isolated nucleic acid of claim 37, which encodes the antibody heavy  
20 chain variable region and an antibody heavy chain constant region.

39. The isolated nucleic acid of claim 38, wherein the antibody heavy chain constant region is an IgG1 constant region.

25 40. The isolated nucleic acid of claim 38, wherein the antibody heavy chain constant region is an IgG4 constant region.

41. The isolated nucleic acid of claim 38, which is in a recombinant expression vector.

30

42. A recombinant expression vector encoding:

a) an antibody light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 1; and

b) an antibody heavy chain having a variable region comprising  
35 the amino acid sequence of SEQ ID NO: 2.

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43. A host cell into which the recombinant expression vector of claim 42 has been introduced.

44. A method of synthesizing a human antibody that binds human TNF $\alpha$ ,  
5 comprising culturing the host cell of claim 43 in a culture medium until a human antibody that binds human TNF $\alpha$  is synthesized by the cell.

45. A pharmaceutical composition comprising the antibody, or antigen-binding portion thereof, of any of claims 1-24, and a pharmaceutically acceptable  
10 carrier.

46. The pharmaceutical composition of claim 45, which further comprises at least one additional therapeutic agent for treating a disorder in which TNF $\alpha$  activity is detrimental.  
15

47. A method for inhibiting human TNF $\alpha$  activity comprising contacting human TNF $\alpha$  with the antibody, or antigen-binding portion thereof, of any of claims 1-24 such that human TNF $\alpha$  activity is inhibited.

48. A method for inhibiting human TNF $\alpha$  activity in a human subject suffering from a disorder in which TNF $\alpha$  activity is detrimental, comprising administering to the human subject the antibody, or antigen-binding portion thereof, of any of claims 1-24 such that human TNF $\alpha$  activity in the human subject is inhibited.  
20

49. The method of claim 48, wherein the disorder is sepsis.  
25

50. The method of claim 49, wherein the antibody is administered to the human subject together with the cytokine interleukin-6 (IL-6) or is administered to a human subject with a serum or plasma concentration of IL-6 above 500 pg/ml.  
30

51. The method of claim 48, wherein the disorder is an autoimmune disease.

52. The method of claim 51, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis  
35 and gouty arthritis.

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53. The method of claim 51, wherein the autoimmune disease is selected from the group consisting of an allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome.

5 54. The method of claim 48, wherein the disorder is an infectious disease.

55. The method of claim 48, wherein the disorder is transplant rejection or graft-versus-host disease.

10 56. The method of claim 48, wherein the disorder is a malignancy.

57. The method of claim 48, wherein the disorder is a pulmonary disorder.

58. The method of claim 48, wherein the disorder is an intestinal disorder.

15

59. The method of claim 48, wherein the disorder is a cardiac disorder.

60. The method of claim 48, wherein the disorder is selected from the group consisting of inflammatory bone disorders, bone resorption disease, alcoholic hepatitis, viral hepatitis, fulminant hepatitis, coagulation disturbances, burns, reperfusion injury, keloid formation, scar tissue formation, pyrexia, periodontal disease, obesity and radiation toxicity.

20 61. Use of the antibody, or antigen-binding portion thereof, of any of claims 1-24 in the manufacture of a medicament for the treatment of a disorder in which  $\text{TNF}\alpha$  activity is detrimental.

62. The use of claim 61, wherein the disorder is sepsis.

30 63. The use of claim 62, wherein the antibody is administered to the human subject together with the cytokine interleukin-6 (IL-6) or is administered to a human subject with a serum or plasma concentration of IL-6 above 500 pg/ml.

35 64. The use of claim 61, wherein the disorder is an autoimmune disease.

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65. The use of claim 64, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis.

5           66. The use of claim 64, wherein the autoimmune disease is selected from the group consisting of an allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome.

10           67. The use of claim 61, wherein the disorder is an infectious disease.

68. The use of claim 61, wherein the disorder is transplant rejection or graft-versus-host disease.

15           69. The use of claim 61, wherein the disorder is a malignancy.

70. The use of claim 61, wherein the disorder is a pulmonary disorder.

71. The use of claim 61, wherein the disorder is an intestinal disorder.

20           72. The use of claim 61, wherein the disorder is a cardiac disorder.

73. The use of claim 61, wherein the disorder is selected from the group consisting of inflammatory bone disorders, bone resorption disease, alcoholic hepatitis, viral hepatitis, fulminant hepatitis, coagulation disturbances, burns, reperfusion injury, 25 keloid formation, scar tissue formation, pyrexia, periodontal disease, obesity and radiation toxicity.

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	CDR L1		CDR L2
2SD4 VL	DIQMTQSPSSLSASIGDRVTITC	WYQQKPGKAPKLLIY	AASTLQS
EP B12	.....	.....	.....
VL10E4	.....	.....	.....
VL100A9	.....	.....	.....
VL100D2	.....	.....	.....
VL10F4	.....	.....	.....
LOE5	.....	.....	.....
VLL0F9	.....	.....	.....
VLL0F10	.....	.....	.....
VLL0G7	.....	.....	.....
VLL0G9	.....	.....	.....
VLL0H1	.....	.....	.....
VLL0H10	.....	.....	.....
VL1B7	.....	.....	.....
VL1C1	.....	.....	.....
VL1C7	.....	.....	.....
VL0.1F4	.....	.....	.....
VL0.1H8	.....	.....	.....
LOE7	.....	.....	.....
LOE7.A	.....	.....	.....
LOE7.T	.....	.....	.....
D2E7 VL	DIQMTQSPSSLSASVGDRVTITC	WYQQKPGKAPKLLIY	AASTLQS
LD2E7*.A1	.....	.....	.....
LD2E7*.A3	.....	.....	.....
LD2E7*.A4	.....	.....	.....
LD2E7*.A5	.....	.....	.....
LD2E7*.A7	.....	.....	.....
LD2E7*.A8	.....	.....	.....

Figure 1A

		CDR L3																			
		1	2	3	4	5	6	7	8	9		1	2	3	4	5	6	7	8	9	
2SD4 VL	GVPSRFSGSGSGTDFTLT	ISSLQPEDVATYYC	QKYN	SAPYA	FGQGT	KVEIK															
EP B12				R																	
VL10E4				QR																	
VL100A9				S																	
VL100D2																					
VL10F4				R																	
LOE5																					
VLLOF9				R																	
VLLOF10																					
VLLOG7																					
VLLOG9				T																	
VLLOH1				R																	
VLLOH10																					
VL1B7				Q																	
VL1C1																					
VL1C7																					
VL0.1F4				I																	
VL0.1H8				RP																	
LOE7				R																	
LOE7.A				R																	
LOE7.T				R																	
D2E7 VL	GVPSRFSGSGSGTDFTLT	ISSLQPEDVATYYC	QRYN	RAPYT	FGQGT	KVEIK															
LD2E7*.A1				A																	
LD2E7*.A3				A																	
LD2E7*.A4				A																	
LD2E7*.A5				A																	
LD2E7*.A7				A																	
LD2E7*.A8				A																	

Figure 1B



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	CDR H1		CDR H2
2SD4 VH	QVQLVESGGGLVQPGRSRLRLSCAASGFTFD		AITWNSGHI DYADSV EG
VH1B11	.....	WVRQAPGKGLDWVS	.....
VH1D8	.....	.....	.....
VH1A11	.....	.....	.....
VH1B12	.....	.....	.....
VH1-D2	.....	.....	.....
VH1E4	.....	.....	.....
VH1F6	.....	.....	.....
VH1G1	.....	.....	.....
3C-H2	.....	.....	.....
VH1-D2.N	.....	.....	.....
VH1-D2.Y	.....	.....	.....
D2E7 VH	EVQLVESGGGLVQPGRSRLRLSCAASGFTFD	WVRQAPGKGLEWVS	AITWNSGHI DYADSV EG
HD2E7*.A1	.....	.....	.....
HD2E7*.A2	.....	.....	.....
HD2E7*.A3	.....	.....	.....
HD2E7*.A4	.....	.....	.....
HD2E7*.A5	.....	.....	.....
HD2E7*.A6	.....	.....	.....
HD2E7*.A7	.....	.....	.....
HD2E7*.A8	.....	.....	.....
HD2E7*.A9	.....	.....	.....

Figure 2A

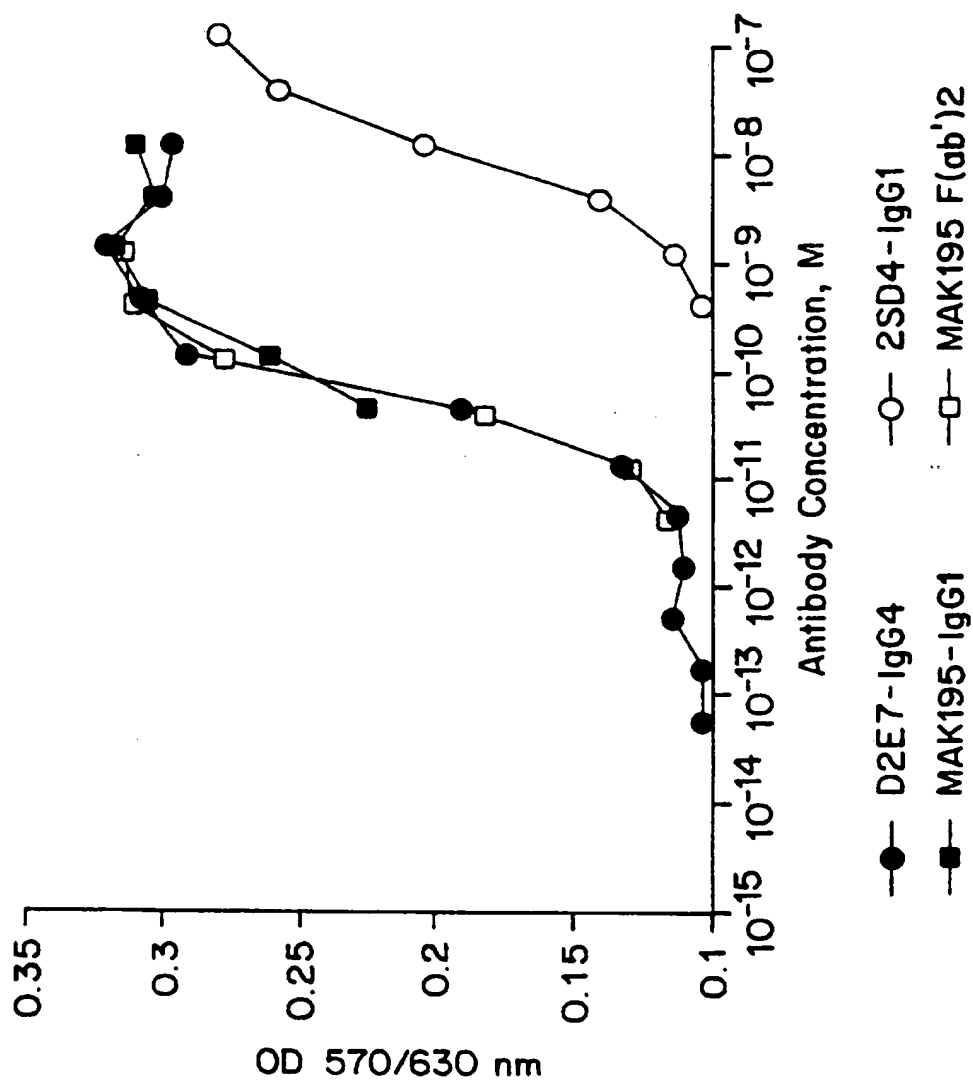
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		CDR H3																										
		1	2	3	4	5	6	7	8	9	10	11	12															
2SD4 VH	RFAVSRD	N	A	K	N	A	L	Y	L	Q	M	N	S	L	R	P	E	D	T	A	V	Y	Y	C	T	K		
VH1B1														K													W	
VH1D8														Y													Q	
VH1A11														D													G	
VH1B12														Y													T	
VH1-D2														F													L	
VH1E4														A													V	
VH1F6														H													W	
VH1G1														Y													Q	
3C-H2														E													G	
VH1-D2.N														Y													T	
VH1-D2.Y														N													V	
D2E7 VH	RFTISR	D	N	A	K	N	S	L	Y	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C	A	K	
HD2E7*.A1														Y													W	
HD2E7*.A2														D													Q	
HD2E7*.A3														A													G	
HD2E7*.A4														Y													T	
HD2E7*.A5														F													L	
HD2E7*.A6														A													V	
HD2E7*.A7														H													W	
HD2E7*.A8														Y													Q	
HD2E7*.A9														N													T	
														Y													V	
																											W	

Figure 2B

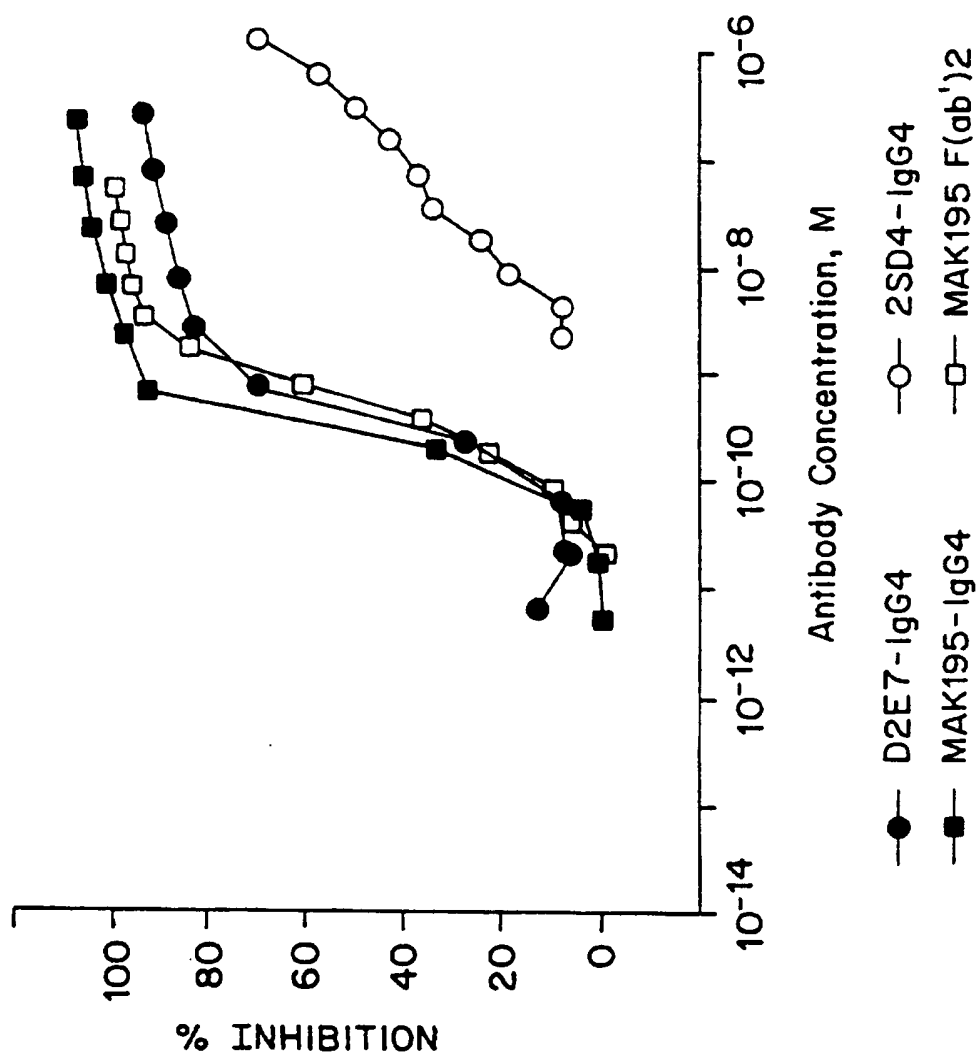
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FIG. 3



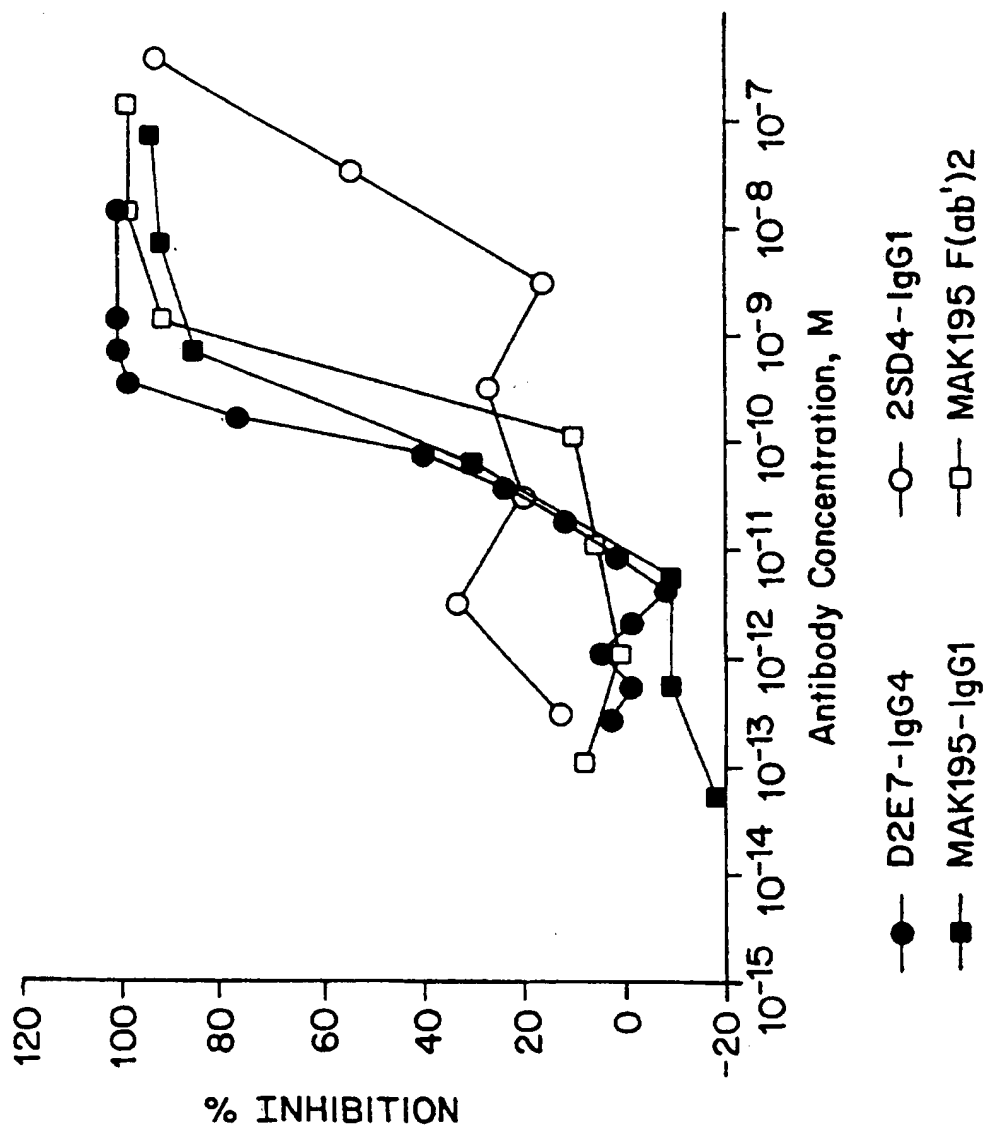
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FIG. 4



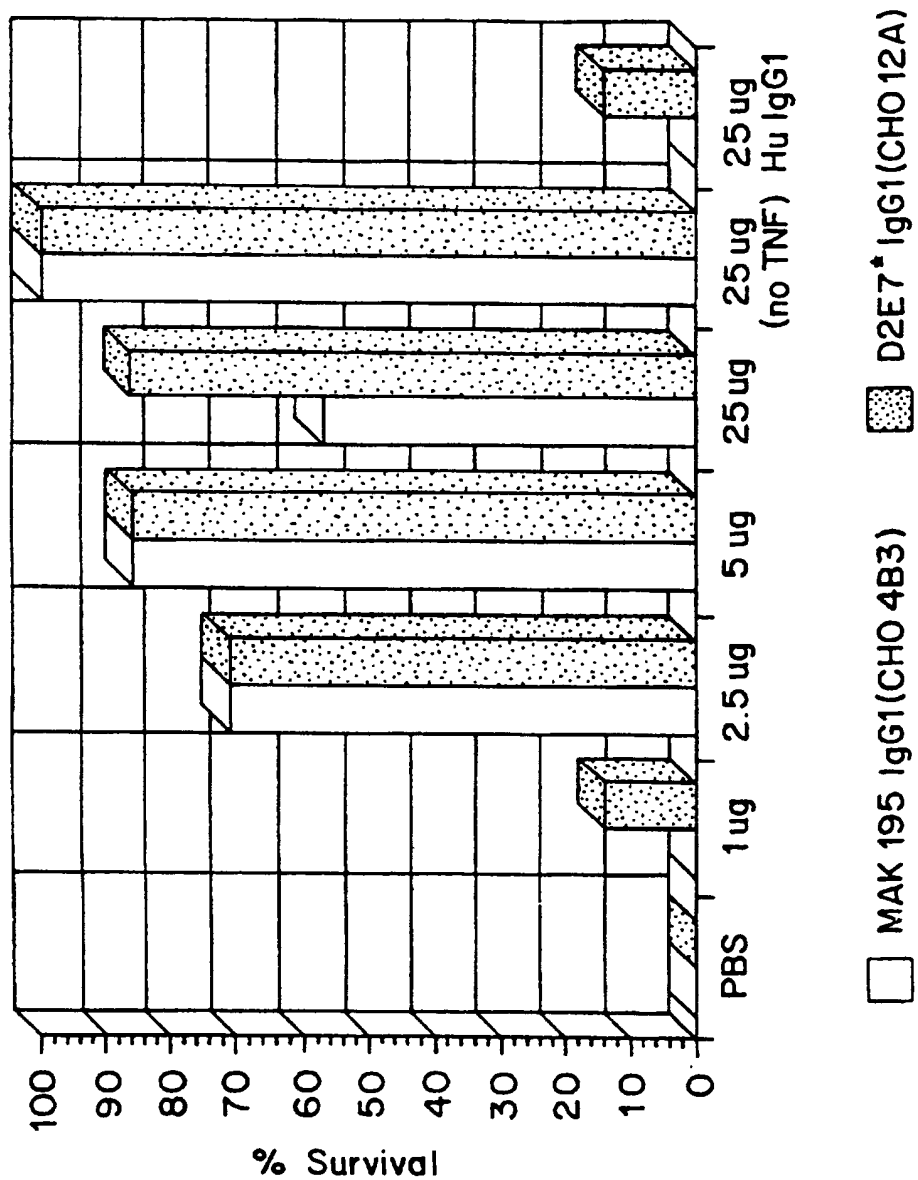
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FIG. 5



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FIG. 6



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D2E7 VL

GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA  
 D I Q M T Q S P S S L S A S V

CDR L1

GGG GAC AGA GTC ACC ATC ACT TGT CGG GCA AGT CAG GGC ATC AGA  
 G D R V T I T C R A S Q G I R

AAT TAC TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG  
N Y L A W Y Q Q K P G K A P K

CDR L2

CTC CTG ATC TAT GCT GCA TCC ACT TTG CAA TCA GGG GTC CCA TCT  
 L L I Y A A S T L Q S G V P S

CGG TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC  
 R F S G S G S G T D F T L T I

AGC AGC CTA CAG CCT GAA GAT GTT GCA ACT TAT TAC TGT CAA AGG  
 S S L Q P E D V A T Y Y C Q R

CDR L3

TAT AAC CGT GCA CCG TAT ACT TTT GGC CAG GGG ACC AAG GTG GAA  
Y N R A P Y T F G Q G T K V E

ATC AAA  
 I K

FIGURE 7

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D2E7 VH

GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTA CAG CCC GGC  
 E V Q L V E S G G G L V Q P G

AGG TCC CTG AGA CTC TCC TGT GCG GCC TCT GGA TTC ACC TTT GAT  
 R S L R L S C A A S G F T F D

**CDR H1**

GAT TAT GCC ATG CAC TGG GTC CGG CAA GCT CCA GGG AAG GGC CTG  
D Y A M H W V R Q A P G K G L

**CDR H2**

GAA TGG GTC TCA GCT ATC ACT TGG AAT AGT GGT CAC ATA GAC TAT  
 E W V S A I T W N S G H I D Y

GCG GAC TCT GTG GAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC  
A D S V E G R F T I S R D N A

AAG AAC TCC CTG TAT CTG CAA ATG AAC AGT CTG AGA GCT GAG GAT  
 K N S L Y L Q M N S L R A E D

**CDR H3**

ACG GCC GTA TAT TAC TGT GCG AAA GTC TCG TAC CTT AGC ACC GCG  
 T A V Y Y C A K V S Y L S T A

TCC TCC CTT GAC TAT TGG GGC CAA GGT ACC CTG GTC ACC GTC TCG  
S S L D Y W G Q G T L V T V S

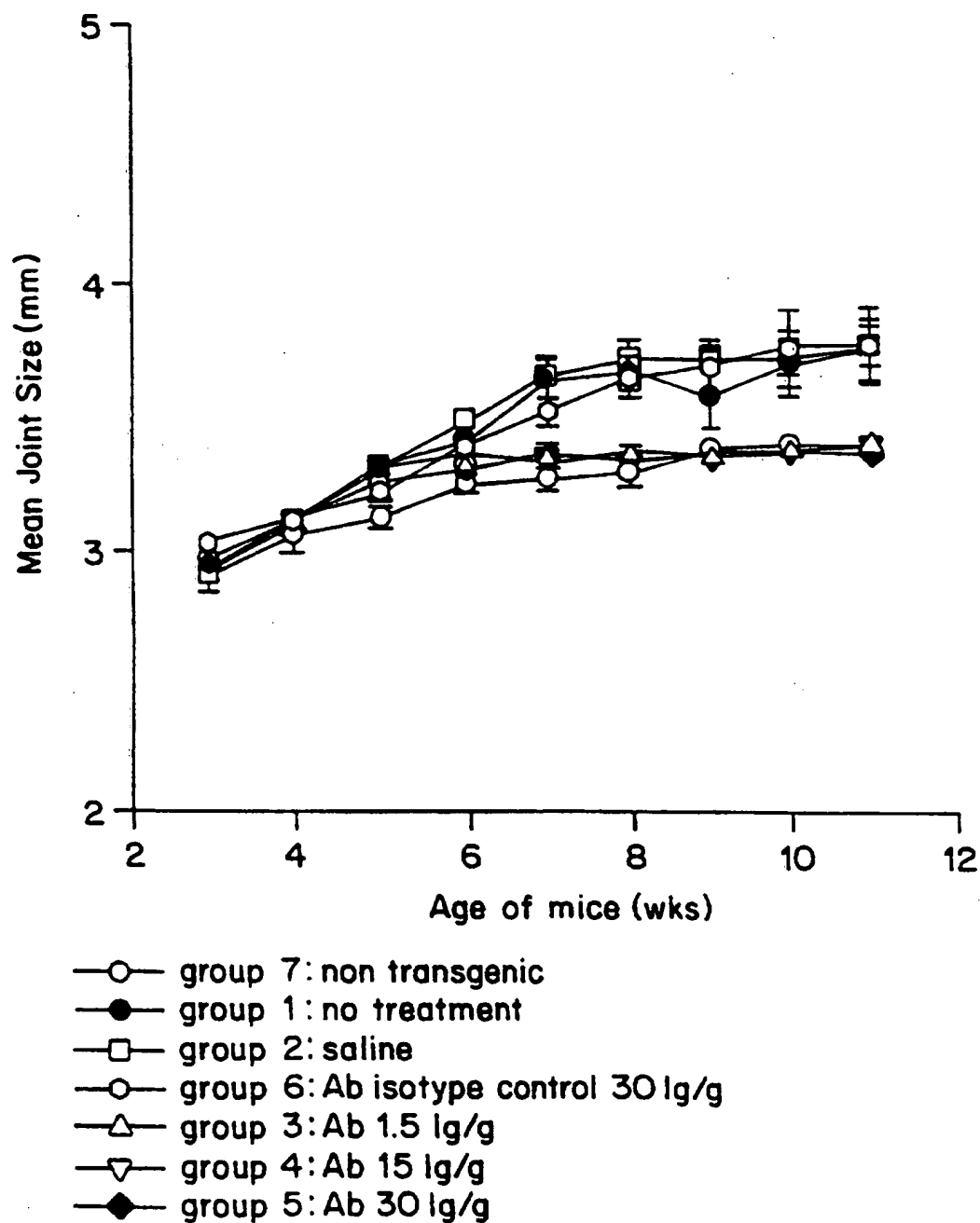
AGT  
 S

FIGURE 8



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FIG. 9



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 97/02219

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K16/24 C12N15/13 C12N15/64 C12N5/10 C12N1/21  
A61K39/395 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 06213 A (MEDICAL RES COUNCIL ;CAMBRIDGE ANTIBODY TECH (GB)) 1 April 1993 cited in the application	1-40
Y	see examples see the whole document ---	41-73
X	EMBO J., vol. 12, no. 2, 1993, pages 725-734, XP000645476 GRIFFITH ET AL.: "Human anti-self antibodies with high specificity for phage display libraries" cited in the application	1-40
A	see the whole document --- -/-	41-73

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"A" document member of the same patent family

Date of the actual completion of the international search

9 June 1997

Date of mailing of the international search report

25.06.97

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

Hagenmaier, S

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No  
PCT/US 97/02219

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 614 984 A (MILES INC) 14 September 1994 cited in the application	1-8
A	see the whole document ---	9-73
Y	WO 92 16553 A (UNIV NEW YORK ;CENTOCOR INC (US)) 1 October 1992 see the whole document ---	41-73
A	GB 2 279 077 A (CELLTECH LTD) 21 December 1994 see the whole document ---	1-73
A	CLINICAL RESEARCH, vol. 42, no. 2, April 1994, page 299a XP002032300 CHOW ET AL.: "Effect of monoclonal antibody to human TNF on TNFalpha, IL-1beta and IL-6 levels in patients with sepsis syndrome" see the whole document -----	50,63

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/02219

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 47 (partially), 48-60  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 47 (partially, as far as an in vivo method is concerned) and 48-60 (all completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/02219

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## INTERNATIONAL SEARCH REPORT

### Information on patent family members

In                      Additional Application No                     

PCT/US 97/02219

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